THE DIFFERENTIAL STAINING OF HUMAN AND DOG SPERMATOZOA

by R. G. WALES

(From the Department of Veterinary Physiology, University of Sydney, Sydney, Australia).

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SUMMARY.

Human spermatozoa can be stained successfully with trypan blue, eosin and erythrosin and although each stain gives a difference in the estimate of "live" cells in a sample, this difference is consistent for each ejaculate, before and after heat treatment.

It is suggested that because of the relative simplicity of the procedure, eosin-nigrosin should be used routinely to stain human spermatozoa.

Eosin-nigrosin should be used in preference to congo red-nigrosin as a vital stain for dog spermatozoa as it gives better differentiation, a variance estimate which is more consistent with that expected from theory and less difference between operators.

The percentage of stained spermatozoa in a semen-eosin mixture has been found to increase with time of exposure to the stain. This effect was seen with both dog and human spermatozoa.

INTRODUCTION.

The introduction of vital stains to determine the proportion of living and dead spermatozoa (Lasley, Easley and McKenzie, 1942) has proved a valuable aid in the evaluation of semen. A considerable literature has accumulated on the use of eosin-nigrosin as a vital stain for ram, bull, boar and stallion semen since its introduction by Blom (1950) and Hancock (1951). Emmens (1947) used congo red as a vital stain and more recently Blackshaw (1958a) has shown that congo red-nigrosin may be used to replace eosin-nigrosin as a vital stain for ram and bull spermatozoa.

Revector soluble blue (Crooke and Mandl, 1947) and eosin (MacLeod, 1942; Mayer, Squiers, Bogart and Oloufa, 1951) both act as vital stains for human spermatozoa and Blackshaw (1958b) has found that trypan blue can replace revector soluble blue in the Crooke and Mandl procedure. However, there is apparently no mention in the literature of the use of vital stains for dog spermatozoa.

The purpose of this paper is to compare the use of trypan blue, eosin and erythrosin for human spermatozoa and to present some observations on the use of eosin and congo red to determine the viability of dog spermatozoa.

Materials and Methods.

Normal human ejaculates were obtained from a sterility clinic. The sperm-bearing fraction and prostatic fluid were obtained from dogs by manipulation.

Trypan blue was prepared and used according to the procedure of Crooke and Mandl (1947). The other semen stains were prepared by dissolving 5 p.c. water soluble nigrosin (Gurr) in 2·6 p.c. sodium citrate dihydrate and adding 3 p.c. congo red or 0·5 p.c. eosin or 0·5 p.c. erythrosin. One or two drops of semen were added to an appropriate quantity of stain, mixed well and allowed to stand for five minutes at room temperature before a thin smear was made on a clean slide. The ratio of semen to stain was altered so that there was always a reasonable number of spermatozoa in a microscopic field.

In some tests, samples of the same ejaculate were used fresh and after immobilising the spermatozoa by immersion in a water bath at 55° C. for 15 min.

For each count, 100 spermatozoa were examined under the microscope and the angular transformation was applied to the percentage of unstained spermatozoa (Fisher and Yates, 1953; Campbell, Hancock and Rothschild, 1953). Since Emmens (1947) noted that eosin showed a tendency to diffuse into hitherto unstained cells after a few hours, eosin-nigrosin and erythrosin-nigrosin smears were always examined soon after preparation.

RESULTS.

Experiments with human semen.

Five ejaculates were stained with trypan blue, eosin-nigrosin and erythrosin-nigrosin, fresh and after immobilising by heat. Two samples of each ejaculate were added to the stains, two smears were made from each sample and one count was made on each smear. The mean percentages of unstained spermatozoa counted for each stain are shown in Table 1, with the analysis of variance in Table 2. The second order interaction mean square which was significantly larger than the between sample or between smear mean squares has been used to test those above it in the Table.

TABLE 1.

The mean percentage of unstained human spermatozoa using trypan blue, eosin and erythrosin as vital stains.

	Ejaculate					Mean
	1	2	3	4	5	
Fresh spermatozoa						
Trypan blue	73·3	71·8	46·8	85·5	51·5	65·8
Eosin	76·8	80·3	64·5	88·3	49·3	71·8
Erythrosin	77·0	81·8	74·0	88·8	58·5	76·0
Heat immobilised spermatozoa						
Trypan blue	15·0	17·0	35·3	77·0	37·5	36·4
Eosin	22·3	22·5	40·8	69·0	42·0	39·3
Erythrosin	31·3	28·8	48·3	86·0	39·8	46·8

TABLE 2.

Analysis of variance for the data of Table 1.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Effect of heat immobilisation	1	10,716	10,716	282·0**
Stain differences	2	1,011	506	13·3**
Ejaculate differences	4	8,713	2,178	57·3**
Interactions:				
Heating/Stain	2	34	17	0·4
Heating/Ejaculate	4	4,388	1,097	28·9**
Stain/Ejaculate	8	294	37	1·0
Heating/Stain/Ejaculate (error 2)	8	305	38	4·3**
Between samples	30	307	10·2	1·11
Between smears (error 1)	60	526	8·8	
Theoretical	∞	—	8·2	

** $P < 0.01$

The estimates of live spermatozoa obtained by the different stains differed significantly: trypan blue gave the lowest percentage of unstained spermatozoa, erythrosin the highest and eosin was intermediate. However, there were no interactions between stains and heat treatment or stains and ejaculates. Ejaculates differed in their reaction to heat, but the fall in the percentage of unstained cells was significant when the mean square for the effect of heating was tested against the large heating X ejaculate mean square ($F = 9{\cdot}8$, $P < 0{\cdot}05$).

The between-smear variance estimates for each stain were 14·3 (trypan blue), 7·1 (eosin), and 4·9 (erythrosin). Bartlett's test showed that these variances were homogeneous ($\chi^2_{(2)} = 5{\cdot}867$, $P > 0{\cdot}05$) and the pooled mean square of 8·8 compares well with the theoretical estimate of 8·207 ($\chi^2_{(60)} = 64{\cdot}3$, $P > 0{\cdot}05$).

TABLE 3.

The mean percentage of unstained dog spermatozoa and the between duplicates mean squares, with and without the addition of prostatic fluid.

	Stain			
	Congo red		Eosin	
	No prostatic fluid	Prostatic fluid	No prostatic fluid	Prostatic fluid
Fresh spermatozoa	79·9	78·6	92·2	90·2
Heat immobilised spermatozoa	36·0	24·5	25·8	20·2
Between duplicates mean square	25·0	76·6	15·1	26·3

TABLE 4.

Analysis of variance for the data of Table 3.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Effect of heat immobilisation	1	31,761	31,761	251·9**
Stain differences	1	58	58	0·5
Effect of prostatic fluid	1	231	231	1·8
Ejaculates	4	4,270	1,068	8·5**
1st order interactions:				
Heating/Stains	1	979	979	7·8*
Heating/Prostatic fluid	1	58	58	0·5
Heating/Ejaculate	4	2,335	584	4·6**
Stains/Prostatic fluid	1	1	1	0·0
Stains/Ejaculate	4	811	203	1·6
Prostatic fluid/Ejaculate	4	1,450	363	2·9
Higher order interactions (error 2)	17	2,143	126	3·5**
Between duplicates (error 1)	40	1,431	36	
Theoretical	∞	—	8·2	

** $P < \cdot 01$
* $P < \cdot 05$

Experiments with dog semen.

Dog spermatozoa were stained with eosin and congo red before and after immobilising by heat as before. To test whether prostatic fluid influences the staining reaction, the sperm-bearing fraction was used with and without the addition of an equal volume of prostatic fluid. Single counts on duplicate smears were made for each treatment and the results are summarised in Table 3 with the analysis of variance in Table 4. The higher order interaction mean square has been used as error as it was significantly larger than the between duplicate mean square. There was no overall difference between stains; eosin gave a higher percentage of unstained spermatozoa in fresh semen, but a lower percentage after heating. As with human semen, the effect of heat differed in each ejaculate but always increased significantly the percentage of unstained spermatozoa even if the heating X ejaculate interaction was used as the error term ($F = 54 \cdot 4$, $P < \cdot 01$). The mean squares for duplicates with congo red and eosin, with and without prostatic fluid, are shown in Table 3. When congo red was used, the presence of prostatic fluid significantly increased the between duplicate variance. With eosin, however, the increase in variance when prostatic fluid was present did not test as significant.

Further tests were undertaken with fresh dog semen to assess operator differences. Five ejaculates were used in the test and for each ejaculate, three semen-stain subsamples were made for each stain, three smears were made from each subsample and each operator made three counts on each smear, each count being made independently of the preceding ones. The mean percentages of unstained cells counted by each operator are shown in Table 5. In the analysis,

the count classification is divided hierarchically into counts, smears, subsamples and replicates, each term in the hierarchy being tested against the one immediately below it. Since congo red gave a significantly larger variance than eosin at each level of the hierarchy, separate analyses were made for each stain (Table 6). Eosin gave between subsample, slide and count variances which were consistent with random sampling, but with congo red, there were highly significant differences between slides from the same subsample.

The analysis showed that there were larger operator differences when congo red was used as stain. The between-counts variance with congo red for operator No. 2 was larger than expectation ($\chi^2_{(90)} = 160$, $P < 0{\cdot}01$) while that for opera-

TABLE 5.

The mean counts and between count mean squares for two operators using congo red and eosin as vital stains for dog spermatozoa.

Ejaculate	Operator			
	1		2	
	Congo red	Eosin	Congo red	Eosin
1	81·6	76·0	78·8	75·6
2	81·4	75·6	81·9	78·2
3	83·7	77·7	80·8	79·6
4	92·9	86·6	95·0	84·9
5	77·0	65·7	86·6	68·2
Mean	83·3	76·3	84·6	77·3
Count mean square	9·9	9·4	14·6**	8·7
Theoretical	8·2	8·2	8·2	8·2

** Significantly larger than theoretical $P < {\cdot}01$.

TABLE 6.

Summary analyses of variance for the data of Table 5.

Source of variation	Degrees of freedom	Congo red		Eosin	
		Mean square	F	Mean square	F
Operators	1	178	8·5**	6	0·7
Ejaculates	4	1,492	71·0**	1,128	125·3**
Operators/Ejaculates	4	187	8·9**	28	3·1*
Between subsamples (error 3)	20	21·0	0·7	9·2	0·9
Between slides (error 2)	60	31·9	2·6**	10·8	1·2
Between counts (error 1)	180	12·2		9·0	
Theoretical	∞	8·2		8·2	

* $P < {\cdot}05$
** $P < {\cdot}01$

tor No. 1 who had more counting experience was consistent with random sampling ($\chi^2_{(90)} = 108$, $P > 0{\cdot}05$). This difference between operators was not observed when eosin was used and the variance for both operators was not significantly different from the theoretical variance ($\chi^2_{(90)} = 103$ and 95 respectively).

Effect of dilution and staining time.

Because of the need to dilute spermatozoa in physiological experiments the effects of dilution on sampling variation and the proportion of cells stained with eosin were studied. Human semen diluted threefold with Ca-free Ringer phosphate and dog semen similarly diluted with Ringer and with 2·6 p.c. sodium citrate dihydrate were compared with undiluted samples of the same ejaculates. Duplicate smears were made from each semen-stain sample and single counts were made from each smear (Table 7). The only significant difference found was in the percentage of unstained dog spermatozoa after dilution in citrate ($F_{(1,32)} = 5{\cdot}3$, $P < 0{\cdot}05$). The duplicate mean squares for dog semen in this experiment were significantly larger than expectation, but this may be due to the high proportion of live spermatozoa (Campbell, Dott and Glover, 1956). However, the use of the method of maximum likelihood (Fisher, 1954) rather than empirical angles, did not decrease the variances (Table 7).

TABLE 7.

The effect of dilution on the percentage of unstained human and dog spermatozoa.

	Human		Dog			
	Ringer (10)*		Ringer (10)		Citrate (16)	
	Undiluted	Diluted	Undiluted	Diluted	Undiluted	Diluted
Mean % unstained	78·9	76·9	92·8	90·0	94·0	91·6
Duplicate mean square (empirical angles)	14·1	13·9	20·9	16·4	18·4	23·8
Duplicate mean square (working angles)			19·0	18·6	18·3	22·0

* No. of replications.

Tests with seven human and six dog ejaculates were made to determine the effect of increasing the staining time in eosin-nigrosin from 5 to 15 minutes. The mean percentages of unstained cells at 5 and 15 minutes were 82·0 and 78·0 respectively for human spermatozoa and 94·8 and 87·5 for dog spermatozoa. Comparison by means of the paired *t*-test showed that these differences are significant.

DISCUSSION.

Human spermatozoa can be successfully stained with trypan blue, eosin and erythrosin. Although they give different estimates of the proportion of dead and alive spermatozoa, these differences are consistent for each ejaculate before and after treatment and therefore, would not be important in the choice of stain. However, the Crooke and Mandl (1947) technique which requires fixing, staining and counter-staining is time-consuming, and unless sufficient care is taken, the slides are difficult to score. Even with eosin and erythrosin the smears will, at times, have a granular rather than a homogeneous nigrosin background but the difficulties found in counting such slides can be partially overcome by making thin smears. The ease in preparation of eosin smears makes this stain convenient for routine use.

Blackshaw (1958a) found that congo red could be used with advantage to replace eosin in estimates of "live" ram and bull spermatozoa. Although both these stains will differentiate dog spermatozoa into stained and unstained groups, the results indicate that eosin gives more reliable estimates of the percentage of unstained and presumably live cells. The semen smear prepared with congo red is difficult to evaluate and accuracy with this stain depends to some extent on the experience of the operator, and may be affected by the difficulty in the interpretation of partially stained spermatozoa (Campbell, Dott and Glover, 1956; Dott, 1956).

When using eosin-nigrosin, however, it is important to standardise the time of staining since there is an increase in the percentage of stained spermatozoa as time of contact with the stain is increased. A similar effect was found by Campbell, Dott and Glover (1956) using ram spermatozoa.

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STUDIES ON THE ESTIMATION OF GLYCEROL, FRUCTOSE AND LACTIC ACID WITH PARTICULAR REFERENCE TO SEMEN

by I. G. WHITE

(From the Department of Veterinary Physiology, University of Sydney).

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SUMMARY.

Zinc sulphate and either barium hydroxide or sodium hydroxide are suitable deproteinizing agents for estimating glycerol, fructose or lactic acid in semen.

The stability of the glycerol and lactic acid colour is fairly good. The fructose colour, however, is likely to increase appreciably on standing for an hour unless the tubes are heated for 20 minutes at 80-85° C. instead of the usual 10 minutes.

Potassium metaperiodate is preferable to sodium paraperiodate in the estimation of glycerol as it gives a calibration curve with a steeper slope.

The glycerol method can be used for the estimation of glycerylphosphorylcholine in semen if fructose is first removed by copper-lime treatment.

Barium-zinc extracts of fructose and lactic acid were found to be stable normally for several weeks in a refrigerator. Contamination with yeast organisms can, however, cause a rapid fall in the fructose content.

INTRODUCTION.

Since the discovery that glycerol increases the revival of deep-frozen spermatozoa it has become widely used in artificial insemination (see Emmens and Blackshaw, 1956), and recently it has been shown that ram and bull spermatozoa can metabolize glycerol under aerobic conditions with the formation of fructose and lactic acid (White, Blackshaw and Emmens, 1954; Mann and White, 1956, 1957; White, 1956, 1957). Thus, when glycerol was added to washed spermatozoa suspensions its concentration was found to decrease and fructose and lactic acid accumulated. This paper is concerned with the colorimetric methods used in estimating these substances — all three of which are now known to occur naturally in semen; the glycerol, however, is in the form of a complex, glycerylphosphorylcholine.

EXPERIMENTAL.

Because of the high proteose content of semen, trichloracetic acid is undesirable as a deproteinizing agent (Mann, 1954) and a mixture of zinc sulphate

with either barium hydroxide or sodium hydroxide is preferable in preparing extracts for the determination of glycerol, fructose and lactic acid. The colour developed by the protein-free extract is in each case compared with that of a standard solution of glycerol, fructose or lactic acid treated in exactly the same way as the semen.

Glycerol and glycerylphosphorylcholine.

Glycerol was estimated by the method of Ryley (1955). This is essentially a modification of the technique of Lambert and Neish (1950), in which glycerol is oxidized to formaldehyde by periodate and the excess periodate destroyed by arsenite. The formaldehyde is then estimated colorimetrically by the formation of a complex with chromotropic acid.

The method has proved useful for determining the rate of breakdown of glycerol when added to washed spermatozoal suspensions and is extended here to the estimation of glycerylphosphorylcholine in semen.

General procedure.—Stock standard solutions of glycerol (3 or 4 mg./ml.) were prepared and stored in a refrigerator. After diluting 1 in 10, suitable aliquots (e.g. 0, 1·0, 2·0 and 3·0 ml.) were taken, the volumes adjusted to 3·0 ml., and 0·5 ml. of 5 p.c. (W/V) $ZnSO_4,7H_2O$ and 0·5 ml. of 0·3 N-$Ba(OH)_2$ or 0·5 ml. of 2 p.c. (W/V) $ZnSO_4,7H_2O$ and 0·5 ml. of 0·1 N-NaOH added. The solutions were centrifuged and 0·5 ml. of supernatant used for analysis.

TABLE 1.

Mean values for optical density in the estimation of glycerol, fructose and lactic acid using 0·1-N NaOH-2 p.c. (W/V) $ZnSO_4$, $7H_2O$ and 0·3 N-$Ba(OH)_2$-5 p.c. (W/V) $ZnSO_4$, $7H_2O$ as deproteinizing agents. The number of replications is given in brackets.

Glycerol (4)			Fructose (3)			Lactic Acid (6)		
μg.	0·1 N-NaOH +2 p.c. $ZnSO_4$, $7H_2O$	0·3 N-$Ba(OH)_2$ +5 p.c. $ZnSO_4$, $7H_2O$	μg.	0·1 N-NaOH +2 p.c. $ZnSO_4$, $7H_2O$	0·3 N-$Ba(OH)_2$ +5 p.c. $ZnSO_4$, $7H_2O$	μg.	0·1 N-NaOH +2 p.c. $ZnSO_4$, $7H_2O$	0·3 N-$Ba(OH)_2$ +5 p.c. $ZnSO_4$, $7H_2O$
5·00	·223	·212	50	·145	·164	3·00	·175	·175
10·00	·385	·381	100	·260	·260	6·00	·334	·342
15·00	·545	·542	150	·369	·367	9·00	·486	·499

Deproteinizing agents.—Table 1 shows the results of a series of tests on standard solutions of glycerol, in which colour development is compared using 0·3 N-$Ba(OH)_2$—5 p.c. (W/V) $ZnSO_4,7H_2O$ and 0·1 N-NaOH—2 p.c. (W/V) $ZnSO_4,7H_2O$. Both alkalis gave essentially the same result and a linear relation between concentration and optical density. Barium hydroxide will stain

TABLE 2.

Stability of the colour developed in the estimation of glycerol, fructose and lactic acid. Mean optical density values are given with the number of replications in brackets.

Glycerol (3)					Fructose (4)										Lactic acid (4)				
					Heated 10 min.					Heated 20 min.									
μg.	0	1 Hr.	2 Hr.	3 Hr.	μg.	0	1 Hr.	2 Hr.	3 Hr.	μg.	0	1 Hr.	2 Hr.	3 Hr.	μg.	0	1 Hr.	2 Hr.	3 Hr.
3·75	0·088	·073	·077	·075	50	·167	·174	·175	·168	50	·165	·164	·165	·165	3·00	·163	·173	·186	·168
7·50	·167	·155	·147	·151	100	·301	·338	·331	·326	100	·322	·322	·322	·326	6·00	·321	·333	·327	·326
11·25	·233	·224	·215	·212	150	·446	·489	·503	·499	150	·482	·488	·490	·488	9·00	·490	·499	·498	·495
15·00	·289	·279	·277	·272	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE 3.

Summary of the analysis of variance for the data in Table 2 showing variance ratios with the error mean square in italics at the base of the columns.

	Glycerol		Fructose			Lactic acid	
	D.F.	V.R.	D.F.	V.R. 10 mins.	V.R. 20 mins.	D.F.	V.R.
Between hours:	3	10·2**	3	26·4**	1·8	3	8·2**
Linear	1	23·2**	1	37·1**	1·8	1	4·3
Quadratic	1	6·2*	1	40·7**	0·5	1	20·1**
Cubic	1	1·2	1	1·4	0	1	0·1
Between replications	2	156·8**	3	1·6	54·3**	3	59·8**
Between concentrations	3	1,393·2**	2	5,886·4**	22,630·0**	2	947·6**
Interactions:							
H/R	6	3·4*	9	2·4	3·0*	9	5·1**
H/C	9	1·0	6	2·9*	0·5	6	5·9**
R/C	6	2·4	6	5·6**	2·0	6	2·1
H/R/C	18	*15*	18	*1·4*	*0·4*	18	*44*

* $P<0·05$ ** $P<0·01$

pipettes unless they are rinsed immediately and NaOH is more convenient to use routinely, although it may sometimes be necessary to heat the extract in order to precipitate protein completely.

Absorption spectrum of chromogen. – The absorption spectrum of the chromogen was examined with glycerol in a final amount of 7·5 μg. The optical density has been plotted against the wavelength and the curve is shown in Fig. 1. A peak occurs at a wavelength of 580 mμ., and is clearly the most desirable wavelength to use.

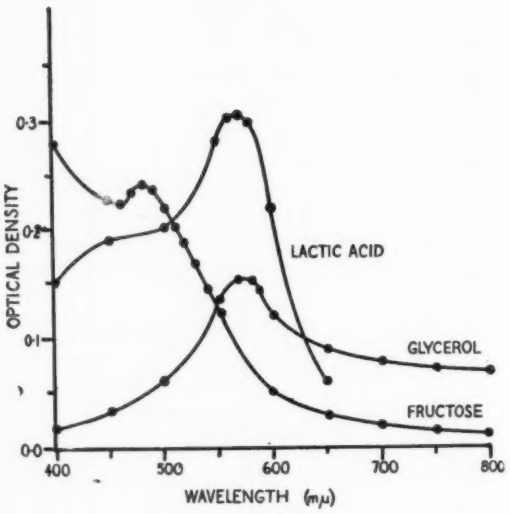

Fig. 1. *Absorption spectra of chromogens in the estimation of glycerol, fructose and lactic acid.*

Stability of chromogen.–The optical density was read immediately on completing the analysis of glycerol, and after the tubes had stood at room temperature 1, 2 and 3 hours. Table 2 shows the results and Table 3 the analyses of variance.

The glycerol samples showed a small but statistically significant decrease in optical density on standing, but this should not seriously affect results, particularly if readings are completed within an hour.

Choice of oxidizing agents in estimation of glycerol.–The reagent used by Ryley (1955) was 0·1 M-sodium metaperiodate ($NaIO_4$). As this salt may not be readily available saturated aqueous solutions of potassium metaperiodate (KIO_4) and sodium paraperiodate (Na_5IO_6) were tried. Table 4 shows the

TABLE 4.

Comparison of sodium paraperiodate and potassium metaperiodate as oxidizing agents for the estimation of glycerol. Each value represents the mean optical density of 5 analyses.

μg. Glycerol	Sodium paraperiodate	Potassium metaperiodate
3·75	0·104	0·123
7·50	0·195	0·242
11·25	0·282	0·347
15·00	0·351	0·464

result for five experiments each involving four concentrations of glycerol. With both salts a linear relationship was obtained between glycerol concentration and optical density but the potassium metaperiodate group has a steeper slope and it is therefore the reagent of choice. In all 25 estimations the potassium metaperiodate gave a higher reading than the sodium paraperiodate when oxidizing the same concentration of glycerol and an analysis of variance showed the difference was highly significant.

Estimation of glycerol and glycerylphosphorylcholine in the presence of fructose.—Periodate oxidation of the glycerol bound in glycerylphosphorylcholine might be expected to yield only one molecule of formaldehyde instead of the two arising from the two terminal hydroxyl groups of the free glycerol molecule (see Dyer, 1956). This was borne out by comparative analyses on glycerol and glycerylphosphorylcholine in which it was found that the glycerol bound in the complex gave only half the colour of the equivalent amount of free glycerol. The estimation of glycerylphosphorylcholine in semen by the periodate method was found to be further complicated by the presence of fructose – a normal constituent of most semen – which also reacts with periodate to give formaldehyde. Ryley (1955) has suggested that glycerol might be estimated in the presence of glucose by preparing standards containing varying amounts of glycerol and sugar. Standard mixtures of glycerol and fructose could presumably be used for the analysis of glycerylphosphorylcholine in semen. Such a procedure would necessitate the estimation of fructose in the semen and its concentration, anyway, is likely to vary during an experimental period due to the breakdown of the sugar to lactic acid.

A more elegant method is to use copper-lime treatment, based on that of Harvey and Higby (1951), prior to the periodate oxidation. To 2·7 ml. of the barium-zinc extract, 0·3 ml. of 20 p.c. (W/V) $CuSO_4,5H_2O$ and 0·42 gm. of $Ca(OH)_2$ were added; the mixture was stirred and allowed to stand for 30 minutes. After centrifuging 0·5 ml. of the supernatant was used for analysis.

Fructose is almost quantitatively removed by this procedure; thus when 10 mg. of fructose were added to 4 ml. barium-zinc extracts containing 0, 300, 600, 900 and 1200 μg. of glycerol respectively, analyses done in triplicate showed that on the average, less than 1 p.c. of the fructose was present in the copper-lime supernatant. Some loss of glycerol may occur owing to absorption on to the copper-lime precipitate, but the amount absorbed is proportional to the glycerol concentration which is still linearly related to the optical density after copper-lime treatment. The value of the copper-lime procedure for the estimation of glycerol in the presence of fructose is illustrated in Table 5 and Fig. 2. These results are from three experiments in which the optical density at four glycerol concentrations has been measured in the presence and absence of fructose (10 mg./4 ml. of barium-zinc extract) with and without copper-lime treatment. In the absence of the copper-lime treatment, fructose seriously inter-

TABLE 5.

Effect of copper-lime treatment on the estimation of glycerol in the presence and absence of fructose. Each value represents the mean optical density of 3 analyses.

Glycerol (μg.)	No copper-lime		Copper-lime	
	No fructose	Fructose	No fructose	Fructose
0	·000	·628	·027	·040
3·75	·201	·703	·219	·205
7·50	·388	·782	·339	·320
11·25	·513	·883	·482	·495
15·00	·658	·980	·603	·595

feres with the estimation of glycerol. The slope of the glycerol curve may be reduced by the addition of copper-lime, but fructose no longer interferes.

Glycerylphosphorylcholine is apparently absorbed on the copper-lime precipitate to the same extent as glycerol and again gave about half the colour that would be expected from its glycerol content when both it and the free glycerol with which it was compared, were subject to copper-lime treatment. It is thus possible to estimate the glycerylphosphorylcholine content of semen by comparing the colour developed on periodate oxidation with standard glycerol solutions which have been treated with barium-zinc and copper-lime in exactly the same way as the semen. In order to express the results as glycerylphosphorylcholine the glycerol values are multiplied by 5·7 (i.e.

$$2 \times \frac{\text{M.W. glycerylphosphorylcholine}}{\text{M.W. glycerol}}).$$

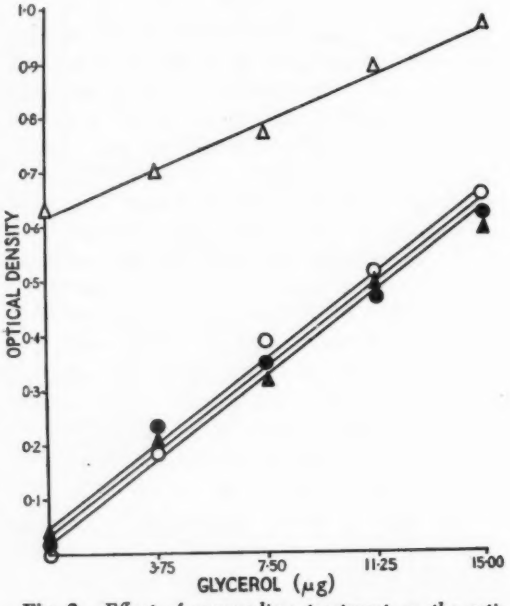

Fig. 2. *Effect of copper-lime treatment on the estimation of glycerol in the presence and absence of fructose* (250 μg.). △—*fructose, no copper-lime;* ▲—*fructose, copper-lime;* ○—*no fructose, no copper-lime;* ●—*no fructose, copper-lime.*

In order to check if other seminal constituents and related substances might interfere, 1 p.c. (W/V) choline chloride, 5 mg. p.c. (W/V) serine, 0·5 p.c. (W/V) sorbitol, 0·25 p.c. (W/V) spermine, 1·0 p.c. (W/V) inositol, 0·5 p.c. (W/V) lactic acid, 20 mg. p.c. (W/V) ascorbic acid and 1 p.c. (W/V) citric acid were prepared. One millilitre of each solution was diluted with 2·0 ml. of water and 0·5 ml. of 5 p.c. (W/V) $ZnSO_4,7H_2O$ and 0·5 ml. of 0·3 N-$Ba(OH)_2$ added. No appreciable colour was found when glycerol was estimated after copper-lime treatment. These substances are not likely to be found in higher concentrations in semen (see Mann, 1954) and the method would thus seem to be fairly specific for glycerylphosphorylcholine in this fluid.

The mean glycerylphosphorylcholine contents of five ram and four bull ejaculates were found to be 1,437 ± 220 (S.D.) mg. p.c. and 305 ± 42 (S.D.) mg. p.c. respectively by this method and are of the same order as the figures quoted by Dawson, Mann and White (1957) using the estimation of acid-labile choline (Appleton, LaDu, Levy, Steel and Brodie (1953)).

Fructose.

Apart from its value in the study of the metabolism of spermatozoa, the estimation of seminal fructose is becoming increasingly important for assessing semen quality in artificial insemination and in the diagnosis of infertility (Mann, 1948 and 1952). Fructose is formed from blood glucose by the accessory sexual organs, of which the seminal vesicles are usually the most important in this regard (Mann, 1954). The concentration varies between animals and even from time to time within individuals and is, in itself, of no value in assessing semen quality. The rate of breakdown, or fructolysis on the other hand, is correlated with the number of motile spermatozoa and probably with fertility (Bishop, Campbell, Hancock and Walton, 1954; Hopwood, Rutherford and Gassner, 1956; Erb, Flerchinger, Ehlers and Gassner, 1956.)

General procedure.—Fructose was estimated by the method of Roe (1934) as modified by Mann (1948). A red colour is produced when the sugar reacts with resorcinol in the presence of hydrochloric acid (Seliwanoff reaction).

A stock standard solution containing 1 mg. of fructose/ml. was used and 5 p.c. (W/V) $ZnSO_4,7H_2O$ – 0·3 N-$Ba(OH)_2$ or 2 p.c. (W/V) $ZnSO_4,7H_2O$ – 0·1 N NaOH added as in the glycerol method. Two millilitres of the extract was used for analysis.

Deproteinizing agents.—The $ZnSO_4$ – $Ba(OH)_2$ and $ZnSO_4$ – NaOH deproteinizing systems gave similar results when tested on the standard fructose solutions (Table 1).

Absorption spectrum of chromogen.—The absorption spectrum of the chromogen was examined with fructose in a final amount of 50 μg. A peak occurs at a wavelength of 480 mμ (Fig. 1). Although it does not seriously matter, there seems little justification for using a wavelength of 530 mμ as is practised in some laboratories in the estimation of seminal fructose.

Stability of chromogen.—In preliminary tests on 16 fructose samples heated at 80-85° C. for the usual 10 minutes, it was found that all tubes showed a considerable increase in optical density after an hour at room temperature. During the second hour there was little further development of colour which faded slightly after a day. The increase in optical density was proportional to the fructose concentration and a linear relation between the two was maintained. If, however, the heating time was increased to 20 minutes no increase in colour occurred on standing. A comparison of the colour stability after heating for 10 and 20 minutes is shown in Tables 2 and 3.

Colour development is apparently not always complete during the shorter heating period and unless all readings are delayed for over two hours any interval between reading standard and unknown fructose should clearly be avoided. When a large number of samples is being analysed the shorter incubation period may give inflated values unless the standards are rechecked during or at the end of the series of unknowns and allowance made for any increase in optical density. If, as is sometimes the practice, a calibration curve is used and no standard fructose solutions are analysed simultaneously with the unknowns it is particularly important to standardise the time of reading rigorously if only 10 minutes is allowed for heating. The 20-minute incubation period is recommended as a routine procedure.

Stability of fructose in protein-free semen extracts.—Four 0·5 ml. samples of bull semen were each diluted with 14·5 ml. of water and 2·5 ml. of 5 p.c. (W/V) $ZnSO_4,7H_2O$ and 2·5 ml. of 0·3 N-$Ba(OH)_2$ added. After centrifuging, 1 ml. of supernatant was used for fructose analysis by comparison with simultaneously prepared standards and the remainder stored in a refrigerator and aliquots taken at weekly intervals for analysis. There was no appreciable difference in fructose content after five weeks, the mean initial and final concentrations being 419 ± 107 (pop. S.D.) and 403 ± 107 (pop. S.D.) mg. p.c. respectively. After six weeks, however, one sample became turbid and microscopic examination showed the presence of numerous yeast cells; one week later the fructose content had fallen to zero. Clearly fructose is normally stable for several weeks in barium-zinc extracts of semen but spurious results are likely to be obtained if micro-organisms proliferate. Turbid extracts should be regarded with suspicion and examined under the microscope to check this point. If extracts are to be kept for long periods, it is advisable to store them in a deep-freeze or to add a wide spectrum antibiotic.

Lactic acid.

Most of the lactic acid found in semen after ejaculation arises from the breakdown of fructose by the spermatozoa, and the estimation of the rate of production of the acid has also been used in assessing semen quality (Mann, 1954). Lactic acid was estimated by a modification of the method of Barker

and Summerson (1941), the lactic acid being oxidised by sulphuric acid to acetaldehyde which gives a coloured compound with p-hydroxydiphenyl.

General procedure.—A stock standard solution of lithium lactate ($\equiv 1{\cdot}2$ mg./ml. lactic acid) was used and 5 p.c. (W/V) $ZnSO_4,7H_2O - 0{\cdot}3$ N-$Ba(OH)_2$ or 2 p.c. (W/V) $ZnSO_4,7H_2O - 1{\cdot}1$ N NaOH added as in the other methods.

One millilitre of the barium-zinc extract was treated with 9·0 ml. of 2·5 p.c. (W/V) $CuSO_4,5H_2O$ and about 1 gm. of $Ca(OH)_2$ added. After centrifuging 1 ml. of the supernatant was used for analysis.

Deproteinizing agents.—Either barium hydroxide or sodium hydroxide can be satisfactorily used in combination with the appropriate amount of zinc sulphate (Table 1).

Absorption spectrum of chromogen.—This was examined with lactic acid in a final amount of 2 μg. A peak occurs at a wavelength of 565 mμ (Fig. 1).

Stability of chromogen.—There was a small but significant increase in colour after three hours (Tables 2 and 3), but this should not seriously affect results.

Stability of lactic acid in protein-free semen extracts.—Four samples of ram semen were kept at room temperature for three hours to allow the accumulation of lactic acid; the semen was then deproteinized as in the estimation of fructose and 1 ml. aliquots of the stored extract taken for lactic acid analyses, by comparison with simultaneously prepared standards, at weekly intervals. There was no appreciable change in lactic acid content after four weeks, the mean initial and final concentrations being 369 ± 121 (pop. S.D.) and 379 ± 116 (pop. S.D.) mg. p.c. respectively.

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PERMEABILITY OF NORMAL AND INJURED SKIN CAPILLARIES TO LIPOPROTEINS IN THE RABBIT

by F. C. COURTICE

(From the Kanematsu Institute, Sydney Hospital, and the Department of Experimental Pathology, Australian National University, Canberra).

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SUMMARY.

Lymph was collected from the hind paws of rabbits in different types of lipaemia. Hypercholesterolaemia was induced by adding cholesterol to the diet and hyperlipaemia by the intravenous injection of Triton WR-1339.

In these conditions the lipids in the lymph from the hind paw were present as all the lipoprotein complexes, including lipid particles, of the plasma in average concentrations of about 20 p.c. of the plasma levels.

When the skin capillaries were injured by scalding the paw, the lymph became quite milky in appearance when the plasma was milky. After the intravenous infusion of an artificial fat emulsion in which the lipid particles were about 1 μ in diameter, there was only a relatively small increase in these particles in the lymph, which macroscopically remained clear.

The possible mechanisms of transfer of these lipids across the capillary endothelium are discussed.

INTRODUCTION.

The most widely accepted view for the accumulation of lipid in the intima of the aorta and other large arteries in atherosclerosis postulates that the lipid is derived from the circulating plasma (c.f. Page, 1954, 1958). If this is so, the plasma lipids, present as groups or families of lipoproteins (c.f. Oncley, 1958), pass through the vascular endothelium and accumulate in the subendothelial tissue. A little understood basic problem in the aetiology of atherosclerosis is the manner in which the lipoproteins pass from the circulating plasma to the extravascular tissue fluid.

The blood vessels in the adventitia of the aorta penetrate some distance into the media but do not supply the intima which is avascular. It is therefore unlikely that the lipid, which in early lesions first appears in the intima, has filtered through the capillaries of these vasa vasorum. Little is known of the exchange of proteins and lipoproteins across the endothelium of the large arteries. No one has yet devised a technique for studying directly the filtration of fluid and protein, either across the arterial endothelium or across the

endothelium of the capillaries of the vasa vasorum. Poole and Florey (1958) have used histological techniques to show that in the hypercholesterolaemic rabbit, lipid is present in the endothelial cells over a plaque in the aorta and that lipid-laden macrophages pass through this endothelium. Whether this means that the lipid passes through the cells or is transported by macrophages is not yet clear.

The capillary endothelial membrane is basically similar to that of the aorta and arteries. A knowledge of the permeability of capillaries to lipids in different regions of the body might, therefore, help in elucidating this problem. The structure of capillaries has been studied by the use of the electron microscope and found to vary in different tissues (Palade, 1953; Bennett, Luft and Hampton, 1959). While the capillary membrane in the liver and spleen contains large fenestrations or gaps up to several thousand Å in diameter, and the capillaries of the intestine, kidney and some endocrine glands contain intracellular fenestrations up to several hundred Å in diameter, no pores have been observed in the skin capillaries. It was therefore decided to investigate the passage of various lipoproteins across the membrane of the skin capillaries.

To ascertain the composition of tissue fluid in any region, the regional lymph is collected, for it is generally believed that tissue fluid and lymph are of approximately the same composition (c.f. Yoffey and Courtice, 1956). Lymph collected from several regions of the body of normal animals contains the lipoproteins of the plasma but in smaller concentrations. Even the large particles or chylomicrons seem to pass to some extent from the plasma into the tissue fluid and lymph, mainly in the liver, but also in other tissues (Courtice and Morris, 1955; Morris and Courtice, 1956).

In the present investigations lymph has been collected from the hind paw of the rabbit and the lipid composition determined in various lipaemic conditions. The permeability of the skin capillaries has been investigated in the normal state and also after injury induced by a thermal burn. These experiments show that even in skin capillaries, in which the electron microscope detects no pores, large lipoprotein complexes can penetrate the wall, especially after injury, and that the ease of transfer depends, to some extent at least, on the size of the complexes.

Methods.

General procedure. Hutch-bred rabbits of various strains were used as experimental animals. Hypercholesterolaemia was produced by feeding the rabbits on pollard to which had been mixed powdered cholesterol in a blendor. Each rabbit was given daily 75 gm. pollard containing 1 gm. powdered cholesterol. Most young rabbits ate this amount daily and the level of cholesterol in their plasma rose rapidly to high levels within a few days. In our experience many older rabbits did not eat so much of the mixture and their plasma cholesterol levels did not rise so rapidly. Most investigators give cholesterol dissolved in fat. Popják (1946) found, however, that amorphous cholesterol in water was absorbed more readily than cholesterol dissolved in fat. The lipids in the plasma of these animals

is present as β-lipoprotein and as particles visible with dark-ground illumination. These particles are largely cholesterol and cholesterol esters with very little triglyceride (McCandless) and the phospholipid:cholesterol ratio is low, usually about 0·5. Using the analytical ultracentrifuge, Gofman and his co-workers (1950) have characterized the lipid complexes which appear in the plasma of rabbits fed a diet containing cholesterol.

To produce hyperlipaemia in which the lipid was mainly triglyceride with relatively little cholesterol two procedures were adopted. In one group, the surface-active agent Triton WR-1339 was injected intravenously as a 10 p.c. solution in normal saline in a single dose of 400 mg./kg. This produced a lipaemia within a few hours, but the maximum lipaemia was evident in 2 to 3 days (Kellner, Correll and Ladd, 1951). In this condition the plasma lipid is largely particulate with a high content of triglyceride and phospholipid and a phospholipid:cholesterol ratio of about 2. In a second group of animals, hyperlipaemia was produced by slow infusion of an artificial fat emulsion. Two different emulsions were used, the one a 10 p.c. emulsion of coconut fat stabilized by phosphatide and glycerine (Zilversmit *et al.*, 1956) and the other a commercial preparation, "Lipomul", supplied by The Upjohn Company in which 15 p.c. cottonseed oil is stabilized with phosphatide. In these experiments the lipid infused was particulate triglyceride in which the particles were about 1 μ in diameter. With dark-ground illumination, these particles before infusion and in the plasma after infusion appeared to be on the average much larger than the particles in the plasma of cholesterol-fed or Tritonized animals.

Blood samples were obtained from an indwelling polyethylene tube in the carotid artery and infusions were made into a similar tube in the external jugular vein of rabbits anaesthetized with "Nembutal". Lymph from the hind paw draining mainly skin, was collected from ducts adjacent to the external saphenous vein, just above the ankle-joint. As these ducts were too small to cannulate regularly, they were ligated and incised and the lymph collected with a Pasteur pipette as it issued from the incised duct on passive movement or massage of the foot. To promote a greater lymph flow the paw was kept in a water-bath at 43-45° C. This increased blood flow and filtration without damaging the capillary membrane (Courtice, 1946). Lymph was collected in some animals from ducts at the back of the leg just below the popliteal fossa. The procedure of Kellner (1954) was used in which a small piece of 27-gauge needle was tied into the lymphatic vessel and lymph collected through an attached polyethylene tube, size, P.E. 10. The lymph in both cases came mainly from the paw and the results were similar.

Lymph was also collected after increasing the permeability of the skin capillaries by a thermal injury. The hind paw of the anaesthetized rabbit was immersed in water at 70° C. for one minute and thereafter kept at 43-45° C. In these circumstances the injured paw became grossly oedematous and the lymph flow increased many-fold.

Chemical analyses. In plasma and lymph, total cholesterol was determined by the method of Abell *et al.* (1952), phospholipid by the method of Zilversmit and Davis (1950), total esterified fatty acids by the method of Sterne and Shapiro (1953) and plasma proteins by microkjeldahl digestion and nesslerization. Zone electrophoresis was carried out on samples of plasma and of lymph in barbiturate buffer of pH 8·6. Protein patterns were stained with bromophenol blue and lipid patterns with Sudan black, as described by Swahn (1952).

RESULTS.

Plasma and leg lymph lipids in normal, hypercholesterolaemic and hyperlipaemic rabbits.

The lymph from the hind paw was collected for a period of 1 to 2 hours, during which time an average of about 0·5 ml. of lymph was usually obtained.

This procedure was followed in three groups of rabbits: those in the first group on a normal diet of rabbit pellets, those in the second with cholesterol added to their diet and those in the third were given an intravenous injection of Triton WR-1339, two to three days previously.

The lipid levels in the plasma and lymph are shown in Table 1. On the

TABLE 1.

The concentrations of total cholesterol, phospholipid and total esterified fatty acids in three groups of rabbits, (a) normal (b) hypercholesterolaemic due to cholesterol feeding (c) hyperlipaemic, 2 to 3 days after an intravenous injection of 400 mg./Kg. of Triton WR-1339.

Group	Rabbit No.		Total Cholesterol mg. p.c.		Phospholipid mg. p.c.		Total Fatty Acids m Eq./l.	
			Plasma	Lymph	Plasma	Lymph	Plasma	Lymph
(a) Normal	1		27	10	85	35	—	—
	2		35	19	97	50	—	—
	3		45	15	130	37	—	—
	4		52	18	109	72	—	—
	5		60	23	139	60	—	—
	6		100	53	136	74	—	—
	7		119	29	145	42	7·8	3·5
		Mean	63	24	120	53		
(b) Hypercholesterolaemic	1		516	84	173	63	27·6	5·2
	2		585	127	258	107	—	—
	3		600	187	237	86	19·9	4·4
	4		694	153	263	111	—	—
	5		805	164	405	117	37·7	14·1
	6		1110	298	444	165	34·8	10·9
	7		1185	118	449	90	—	—
	8		1703	340	688	205	—	—
		Mean	900	184	365	118		
(c) Hyperlipaemic	1		237	59	460	161	23·0	6·4
	2		300	63	590	184	96·0	19·8
	3		581	60	970	112	—	—
	4		731	158	1228	290	—	—
	5		1000	188	1651	370	198·0	35·0
		Mean	570	106	980	223		

normal diet provided, the plasma cholesterol level averaged 63 mg. p.c. and the phospholipid 120 mg. p.c. Zone electrophoresis showed that in such conditions these lipids were practically all present as α-lipoprotein with a few chylomicrons to be seen with dark-ground illumination. In the lymph from the paw the cholesterol and phospholipid levels were, on the average, 38 and 44 p.c. of the corresponding levels in the plasma and a few chylomicrons were always present. In these lymph samples the total protein concentration averaged 2·7 gm. p.c.

while that of the plasma was 5·6. The lipid gradient as α-lipoprotein from plasma to lymph was therefore slightly greater than the gradient for the total protein. As the size of the α-lipoprotein molecules is not very different from that of albumin and globulin, it would seem likely that these molecules might all pass through the capillary membrane in the same way.

In the hypercholesterolaemic rabbits, various degrees of severity were induced with an average plasma cholesterol level of 900 mg. p.c. The phospholipid on the average was 365 mg. p.c. giving a phospholipid:cholesterol ratio of 0·41. The lymph in these animals was in some cases opalescent and, examined with dark-ground illumination, contained large numbers of particles. Zone electrophoresis, Fig. 1, showed that the same lipid complexes were present in the lymph as in the plasma. The total cholesterol concentration was on the average 184 mg. p.c. or 20 p.c. of the plasma level while the phospholipid concentration was 118 or 32 p.c. of the plasma level giving a phospholipid:cholesterol ratio of 0·64. This suggests that the smaller lipid complexes which have relatively more phospholipid in relation to cholesterol are transferred through the capillary membrane more readily.

In the lipaemia produced by Triton WR-1339, the plasma levels of cholesterol and of phospholipid averaged 570 and 980 mg. p.c. respectively, giving a phospholipid:cholesterol ratio of 1·72. The corresponding levels in the lymph were 106, or 18·6 p.c. of the plasma level, and 223, or 22·7 p.c. of the plasma level, with a phospholipid:cholesterol ratio of 2·10.

These experiments show that the lymph from the paw of a hypercholesterolaemic or a hyperlipaemic rabbit may contain quite a high level of particulate lipid and of lipoproteins which behave electrophoretically like chylomicrons and β-lipoproteins. The gradient from plasma to lymph is slightly lower in the hypercholesterolaemic rabbits than in those made lipaemic with Triton which agrees with the findings of Kellner (1954).

The effect of injury on the permeability of the capillary wall to lipoproteins.

(i) *Hypercholesterolaemic animals.* In these experiments the total protein concentration in the leg lymph rose after scalding from 2·7 gm. p.c. to 4·5 gm. p.c. while the plasma protein fell from 5·5 to 5·0 gm. p.c. The level of total protein in the lymph thus rose from 49 p.c. of the plasma level before scalding to 90 p.c. after scalding.

The lipid concentrations were as shown in Table 2. The levels of total cholesterol, phospholipid and of total fatty acid in the lymph all increased considerably after injury; cholesterol from 21 to 48 p.c. of the plasma level, phospholipid from 33 to 57 p.c. of the plasma level and total esterified fatty acid from 29 to 47 p.c. of the plasma level. This change occurred immediately after scalding when the oedema fluid and lymph flow increased enormously, the levels remaining fairly constant for the ensuing two hours during which

TABLE 2.

Hypercholesterolaemic rabbits. Lipids in plasma and leg lymph before and after scalding. Mean of 7.

	Plasma	Leg Lymph				
			Hours after scalding			
		Before scalding	0-½	½-1	1-1½	1½-2
Cholesterol mg. p.c.	785 (516-1185)	162 (84-298)	376 (206-555)	368 (200-552)	364 (189-525)	360 (200-531)
Phospholipid mg. p.c.	319 (173-449)	105 (63-165)	180 (99-243)	174 (93-245)	172 (93-236)	165 (95-230)
P-lipid: Chol. ratio	0·41	0·65	0·48	0·47	0·47	0·46
T.F.A. m Eq./l	30·0 (19·9-37·7)	8·7 (4·4-14·1)	14·2 (9·2-20·3)	13·9 (8·4-20·9)	13·9 (8·5-20·9)	13·5 (8·6-20·7)

lymph was collected. In these conditions, therefore, while the total protein content in the lymph reached a concentration of 90 p.c. of the plasma value, the lipids rose to about 50 p.c. of the level in the plasma. Zone electrophoresis showed that the lipid was present in the same fractions as in the plasma (Fig. 1).

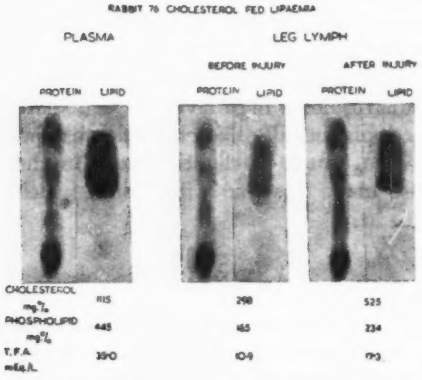

Fig. 1. Electrophoretic patterns, stained for protein and lipid, of the plasma and leg lymph before and after injury of a hypercholesterolaemic rabbit.

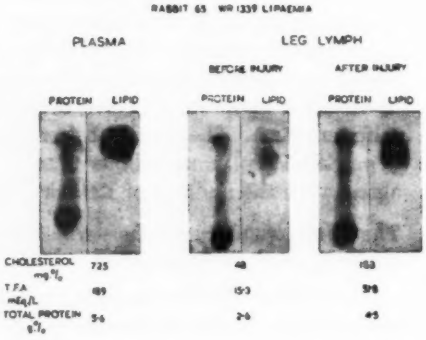

Fig. 2. Electrophoretic patterns, stained for protein and lipid, of the plasma and leg lymph before and after injury of a hyperlipaemic rabbit after WR-1339.

By using dark-ground illumination the number of particles in the lymph was found to be greatly increased, an observation which was also made naked-eye as the lymph became quite milky in appearance.

(ii) *Hyperlipaemic animals.* Similar experiments were performed in rabbits made hyperlipaemic with Triton WR-1339. The average results of three experi-

TABLE 3.

Rabbits with lipaemia due to WR-1339. Lipids in plasma and leg lymph before and after scalding leg. Mean of 3.

	Plasma	Leg Lymph				
			Hours after scalding			
		Before Scalding	0-½	½-1	1-1½	1½-2
Cholesterol mg. p.c.	512 (237-1000)	103 (59-188)	183 (127-286)	201 (121-330)	192 (111-316)	202 (112-344)
Phospholipid mg. p.c.	900 (460-1651)	238 (161-370)	381 (268-544)	389 (268-607)	395 (273-631)	415 (282-678)
P-lipid: Chol. ratio	1·76	2·31	2·08	1·93	2·05	2·05
T.F.A. m Eq./l	105·6 (23·0-198·0)	20·4 (6·4-35·0)	41·3 (19·0-63·0)	44·6 (17·2-73·5)	43·0 (17·5-71·6)	44·7 (16·2-76·7)

ments are shown in Table 3, and six other experiments in which only total cholesterol and total fatty acid concentrations were determined gave similar results. The lipid levels in the lymph all rose considerably after scalding; cholesterol from 20 to 39 p.c. of the plasma level, phospholipid from 26 to 46 and total esterified fatty acid from 19 to 42. These increases were due to a rise in all the lipoprotein fractions observed in plasma, as determined by zone electrophoresis (Fig. 2). The lymph which was often opalescent before scalding became much more milky in appearance and under dark-ground illumination was observed to contain large numbers of particles.

(iii) *Intravenous infusion of hypercholesterolaemic plasma, of hyperlipaemic (Triton WR-1339) plasma and of artificial fat emulsion into rabbits on a normal diet.* Three different types of lipid suspensions were infused into rabbits on a normal diet just after the hind leg was scalded, and the composition of the leg lymph determined during the ensuing 1½ to 2 hours. At the end of this time the oedema fluid in the hind paw (up to the ankle-joint) was determined approximately by weighing postmortem the uninjured and the injured hind paws. In a group of nine rabbits of average weight 3·2 kg., the average amount of oedema fluid was 21 gm. This represents approximately 20 p.c. of the plasma volume. In these circumstances it is unlikely that any appreciable proportion of the escaping lipid would be taken up in adipose tissue in the paw, or that there would be much reabsorption of fluid, so that the plasma:lymph gradient would approximate the plasma:capillary filtrate gradient.

The results of typical experiments with each of the three types of infusion are shown in Fig. 3 and in Tables 4 and 5. When plasma from a hypercholesterolaemic rabbit was infused, the plasma:lymph gradients of cholesterol and of phospholipid (Fig. 3) reached a level about the same as that observed in the

TABLE 4.

Plasma and leg lymph lipids before and after the intravenous infusion of 38 ml. lipaemic plasma from a Tritonized rabbit.

Rabbit ♂ 2·7 Kg.	Plasma			Lymph		
	Total Cholesterol mg. p.c.	Phospholipid mg. p.c.	Total Fatty Acids m Eq./l.	Total Cholesterol mg. p.c.	Phospholipid mg. p.c.	Total Fatty Acids m Eq./l.
Before	100	136	10·7	53	74	5·8
Hind paw scalded at 0 min.						
Infusion of lipaemic plasma 0-30 min.						
0-½ hr.	—	—	—	91	97	9·9
½ hr.	291	434	98·0	—	—	—
½-1 hr.	—	—	—	104	104	18·2
1 hr.	330	490	115·2	—	—	—
1-1½ hr.	—	—	—	116	117	19·2
1½-2 hr.	—	—	—	121	108	19·8
2 hr.	350	510	117·5	—	—	—
Infused plasma	632	890	236			

TABLE 5.

Plasma and leg lymph lipids before and after the intravenous infusion of 20 ml. of 15 p.c. fat emulsion (Lipomul) with the hind paw injured by thermal burn.

Rabbit 23	Plasma		Lymph	
	Phospholipid mg. p.c.	Total Fatty Acids m Eq./l.	Phospholipid mg. p.c.	Total Fatty Acids m Eq./l.
Before	145	7·8	42	3·5
Hind paw scalded at 0 min.				
Infusion of fat emulsion from from 0-80 min.				
0-½ hr.	—	—	68	4·6
½ hr.	238	63·5	—	—
½-1 hr.	—	—	72	4·6
1 hr.	270	88·0	—	—
1-1½ hr.	—	—	70	4·9
1½-2 hr.	—	—	60	4·7
2 hr.	270	82·5	—	—
2-2½ hr.	—	—	62	5·2
2½-3 hr.	—	—	68	5·5
3 hr.	225	50·8	—	—
3-4 hr.	—	—	60	5·7
4 hr.	200	36·1	—	—

injured paw of a chronically hypercholesterolaemic rabbit. The plasma:lymph gradient after the infusion of lipaemic plasma from a Tritonized rabbit (Table 4) was greater than this. When the plasma was made hyperlipaemic by the infusion of an artificial fat emulsion ("Lipomul"), however, there was very little increase in the lipid of the lymph (Table 5). In the experiment represented in Table 5, there was an increase in all the normally present lipid fractions after scalding, but then the effect of the infused fat emulsion was very slight. In this case the fat was present as particles stabilized with phospholipid, so the main increase in the plasma was in total esterified fatty acid and phospholipid. The total esterified fatty acid content of the lymph rose very gradually in four hours and under dark-ground illumination the number of fat particles in the lymph increased. In these experiments, therefore, the lipid passed through the injured skin capillaries most readily when it was present in hypercholesterolaemic plasma and least readily as an artificial fat emulsion. It seemed that the ease with which the lipid was transferred from the plasma to the lymph depended in part at least on the size of the lipid complexes.

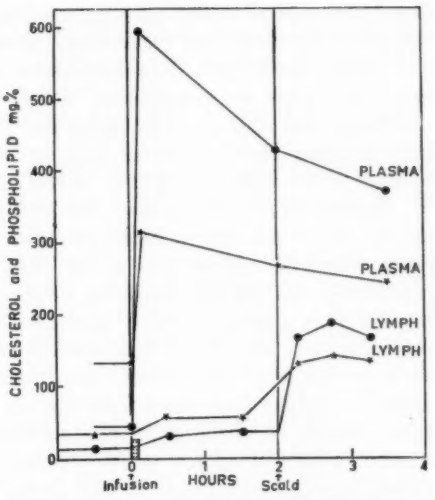

Fig. 3. Rabbit, 2·1 kg. The effect on the levels of lipids in the plasma and leg lymph of intravenous infusion of homologous plasma from a cholesterol fed rabbit and of injury to the leg by thermal burn. From 0-10 minutes, 25 ml. plasma was infused and at 2 hr. the hind paw was scalded. Infused plasma: Cholesterol 1703 mg. p.c. phospholipid 688 mg. p.c.

● Cholesterol.
★ Phospholipid.

DISCUSSION.

In these experiments the concentrations of the various lipid complexes were determined chemically as cholesterol, phospholipid and total esterified fatty acid. While these values might indicate the degree of any one type of lipaemia, it must be realized that these lipids were present in the plasma in complexes of varying size and composition. The results, therefore, which showed that there was a larger plasma:lymph lipid gradient in Tritonized animals compared with hypercholesterolaemic rabbits does not necessarily mean that in Tritonized animals the capillary permeability is decreased as suggested by Kellner (1954). This may be so, but before it could be conclusively proved,

more information would be required concerning the sizes of the various lipid complexes in these two conditions. It would seem from zone electrophoresis and from examination by dark-ground illumination that on the average the lipid complexes were somewhat larger in lipaemia due to Triton than in cholesterol-fed animals. The differences observed in the plasma:lymph gradients may, therefore, be due not so much to any structural difference in the capillary wall as to the difference in size of the lipid complexes.

In normal rabbits in which the lipids were present mainly as the relatively small α-lipoprotein, the concentrations of cholesterol and of phospholipid in the lymph were 38 and 44 p.c. of the plasma levels respectively. In the hypercholesterolaemic rabbit, the lipids were present as complexes which vary greatly in size from α-lipoproteins to particles up to about $0{\cdot}5$ μ in diameter. The plasma:lymph gradient of each individual complex has not been determined in these experiments, but it seems probable that the gradient for the large particles would be greater than that for the smaller complexes. While all lipid complexes were present in the lymph, the cholesterol and phospholipid concentrations were only 20 and 32 p.c. of the plasma levels. In the Tritonized animals the corresponding figures were 18 and 23 p.c. of the plasma levels. It is likely that the α-lipoprotein fraction has the same gradient as in normal animals, but the mean gradient is increased because most of the lipid is present as much larger complexes.

The striking feature of these experiments is the relatively high concentration of the large lipid complexes, such as β-lipoproteins and particles, in the lymph from the paw of hypercholesterolaemic and hyperlipaemic animals and the general relationship between the size of the lipid complex and the plasma: lymph gradient. Further work is required to define this relationship more definitely.

While the pore theory of capillary permeability (c.f. Pappenheimer, 1953; Yoffey and Courtice, 1956) which postulates the presence of pores of 60-90 A in diameter may explain the passage of the smaller lipoprotein complexes, it is difficult to interpret the escape of the larger complexes unless there is a proportion of much larger pores.

Electron microscopy of skin capillaries, however, has failed to show any pores at all. Bennett, Luft and Hampton (1959) have recently classified capillaries according to their structure, as defined by the electron microscope. With regard to pores or fenestrations, capillaries were classified into three groups; the first comprising the sinusoids of the liver and spleen in which large gaps or fenestrations up to several thousand A in diameter were observed, the second comprising the capillaries of the intestinal villi, the kidney and some of the endocrine glands in which were seen intracellular fenestrations or perforations about 200-600 A in diameter, while the third type of capillary, in which the endothelial wall contained no visible fenestrations, was found in skin, muscle, lung and the central nervous system. The endothelial cells of this third type

of capillary were found to contain small vesicles, first described by Palade (1953). It is not known what rôle these vesicles in the endothelial cells play in the transfer of substances across the capillary membrane, but evidence suggests that they are implicated.

This determination of capillary structure by the use of the electron microscope may explain the ready passage of lipid complexes up to the size of chylomicrons through the liver sinusoids, but does not clarify the less ready transfer of these complexes through the skin capillaries as observed in the experiments described above.

If there are no pores or fenestrations in the membranes of the skin capillaries, the large lipid complexes must be transported through the endothelial cells. There are several possible ways of transport which should be considered. Firstly, the large complexes may be broken down at the cell surface, transferred across the cell by some means probably involving the vesicles and reformed into larger complexes again on the other side. If this method of exchange is postulated, the lipid complexes of different size and different composition in the various lipaemic states studied, would be broken down at the endothelial cell surface and re-formed the other side. They would then have to penetrate the endothelium of the lymphatic capillary which probably resembles that of the blood capillary. Another method of transfer might be by macrophages, but it would seem unlikely that macrophages would transfer these lipids across the membrane and then dislodge them into the tissue fluid. It would seem that as far as our present knowledge goes the larger lipid complexes pass through the skin capillaries as such either by way of a relatively few large pores, which have not been revealed by electron microscopy, or by means of a mechanism involving the vesicles.

This view is supported by the experiments described here in which the skin capillaries were injured by heat. The immediate great increase in the passage of lipid particles of different composition would suggest that these particles were transferred as such. In the hypercholesterolaemic and Tritonized animals in which the particles, visible by dark-ground illumination, were small, the oedema fluid in the paw at the end of two hours amounted to about 20 p.c. of the plasma volume and the lipid composition of this fluid was nearly half the plasma level. This rapid transfer of lipid did not apply to the larger particles of an artificial fat emulsion. Certainly the particles of the artificial fat emulsion present in the circulating plasma and in the lymph were larger than the particles in the other lipaemic states investigated. Whether the differences observed in the rate of escape of these lipids across the injured skin capillary membrane were related only to size of the complex has not yet been determined.

Examination by electron microscopy of the endothelium of the skin capillaries in which the permeability was increased by the injection of histamine showed that the vesicles were greatly increased in size, but no actual gaps or fenestrations were observed (Alksne, 1958). Whether this phenomenon is

associated with increased permeability in injury is not known but is suggestive. Although it is possible that these vesicles play a rôle in the active transfer of materials, the experiments described here suggest that there is some physical basis for the transfer of large lipid complexes across the capillary membrane, but the exact nature of the mechanism has not yet been elucidated. These experiments do not give any direct evidence of the transfer of lipids across the aortic endothelium. This membrane has been shown by electron microscopy to contain no visible pores; the endothelial cells, however, contain vesicles similar to those observed in skin capillaries (Buck, 1958; Florey, Poole and Meek, 1959). It would seem, therefore, that structurally the aortic endothelium closely resembles the endothelium of the skin capillaries.

It is reasonable to postulate, therefore, that certain lipoproteins penetrate the aortic endothelium to the subendothelial tissue especially if the wall is injured. If these lipoproteins contained a high proportion of cholesterol, they would, in the absence of lymphatics, probably be taken up by macrophages and remain *in situ* for a considerable time since macrophages dispose of cholesterol very slowly (French and Morris, 1959). Should the macrophages break down the lipid would become an extracellular accumulation.

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THE PERMEABILITY OF LIVER AND SKIN CAPILLARIES TO LIPIDS IN THE CAT

by F. C. COURTICE

(From the Kanematsu Institute, Sydney Hospital, and the Department of Experimental Pathology, Australian National University, Canberra).

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SUMMARY.

Lymph was collected from the hepatic and leg ducts of cats before and after the intravenous infusion of artificial fat emulsions, homologous chyle and heterologous hypercholesterolaemic plasma.

The fat particles of the artificial fat emulsion appeared in the hepatic lymph in quantities similar to those previously found for chyle. In the paw there was only a very little transfer of the particles of artificial fat emulsion and chyle from plasma to lymph even after injuring the skin by a thermal burn. The smaller lipid complexes of hypercholesterolaemic rabbit plasma, however, penetrated the capillary membrane and appeared in the lymph.

The mechanisms of transfer in the liver and in the skin have been discussed.

INTRODUCTION.

The extent of the transfer of the circulating plasma lipids across the capillary endothelium depends in part upon the concentration and the structure of the lipid complexes and in part upon the structure of the endothelium which varies in different tissues. In the liver where the endothelium of the sinusoids has large gaps or deficiences up to several thousand Å in diameter (Bennett, Luft and Hampton, 1959), the smaller α- and β-lipoproteins are readily transferred to the tissue fluid and lymph, and the large chylomicrons rapidly penetrate the sinusoidal membrane and are taken up by the parenchymal cells. Although lipoprotein lipase may accelerate this transfer (c.f. Frederickson and Gordon, 1958), the experiments of Courtice and Morris (1955, 1956) and of French and Morris (1957) led them to postulate that a small proportion of chylomicrons at least passed from the plasma into the hepatic lymph as such, without first being hydrolysed. In these experiments, the quantity of chyle that could be infused raised the plasma lipids for only a short time.

It was hoped that further information would be obtained if, instead of chyle, an artificial fat emulsion was used. This could be readily infused in higher concentration and over a longer period during which time hepatic lymph could be collected and analysed. The concentration of particulate fat in the

lymph would depend on the amount transferred through the sinusoidal membrane and the rate of uptake by the parenchymal cells.

In contrast to the structure of the hepatic sinusoids, the skin capillaries contain no pores or fenestrations visible by electron microscopy. Experiments have shown that in the rabbit, lipoproteins and certain lipid particles penetrate the skin capillaries of the paw, more rapidly when the capillaries are injured by heat (Courtice, 1959). In such a preparation it seems that most of these lipid complexes, having escaped from the circulating plasma through the capillary membrane, would be returned to the blood stream by the lymphatics rather than be taken up by the cells as in the liver. The study of the flow and lipid composition of the lymph, therefore, should be a measure of the extent of lipid transfer across the capillary walls in this region. Experiments have been performed in which artificial fat emulsions and hypercholesterolaemic plasma were infused in the cat. The lymph from the hind paw was collected before and after injury and the extent of transfer of the different lipid complexes determined.

Methods.

Cats of both sexes were used, anaesthetized with Nembutal. A polyethylene catheter was inserted into the external jugular vein through which the fat emulsion was slowly infused by means of a Palmer constant infusion apparatus. Blood samples were obtained from a catheter in the carotid artery.

Hepatic lymph was collected from a polyethylene catheter in the main lymph duct arising from the hilus of the liver (Cain, Grindlay, Bollman, Flock and Mann, 1947). In dogs it has been shown that this duct transports about 80 p.c. of liver lymph (Ritchie, Grindlay and Bollman, 1959).

Lymph from the hind paw was collected by the same procedure as that used in rabbits (Courtice, 1959). In some animals the lymph ducts adjacent to the external saphenous vein just above the ankle-joint were ligated and incised and the lymph collected with a Pasteur pipette; in others the lymph ducts at the back of the leg just below the popliteal fossa were isolated, ligated and one cannulated with a short piece of 25-gauge needle to which a polyethylene tube, size 20, was attached. In either method, lymph was collected on passive movement of the paw with intermittent massage along the ducts. To promote flow, the paw was kept in a water-bath at 43-45° C.

To injure the skin capillaries, the paw was immersed in water at 70° C. for one minute. As with the rabbit, this caused a profuse oedema and a large increase in lymph flow.

Two artificial fat emulsions were infused, 10 p.c. coconut fat or triolein stabilized with phosphatide and glycerine (Zilversmit, Salky, Trumbull and McCandless, 1956) and Lipomul, a 15 p.c. emulsion of cottonseed oil, supplied by The Upjohn Company. As these emulsions did not pass through the injured skin capillaries, thoracic duct homologous chyle and hypercholesterolaemic rabbit plasma were also used.

The analytical methods were the same as those described in a previous paper (Courtice, 1959).

RESULTS.

The effect of intravenous infusions of artificial fat emulsion on hepatic lymph.

When an artificial fat emulsion in relatively large quantities, 3 gm./Kg. body weight within two hours, was infused intravenously, the disappearance

from the circulation was very rapid, but the plasma concentration was maintained at a fairly high level for three hours or more. Fig. 1 represents a typical experiment. The level of esterified fatty acids in the plasma rose in this experiment from 8·1 to 54·4 m Eq. per litre and was maintained at levels fluctuating from 30·6 to 48·8 m Eq. per litre. In the hepatic lymph the level rose from 7·4 to 15·9 m Eq. per litre and with dark-ground illumination there appeared to be an obvious increase in the number of particles present. Of the 3 gm./Kg. fat infused, it was calculated from the plasma concentrations and an assumed plasma volume of 50 ml. per kg. body weight, that about 85 p.c. had disappeared from the circulation within the 3½ hours, of which only 0·6 p.c. was present in the hepatic lymph. Two other experiments gave similar results. The results of these experiments with large infusions of artificial emulsions thus resemble those obtained when homologous chyle was infused (Morris and Courtice, 1956; French and Morris, 1957). It appeared that even with such a large infusion, the disappearing fat was rapidly taken up by the parenchymal cells of the liver.

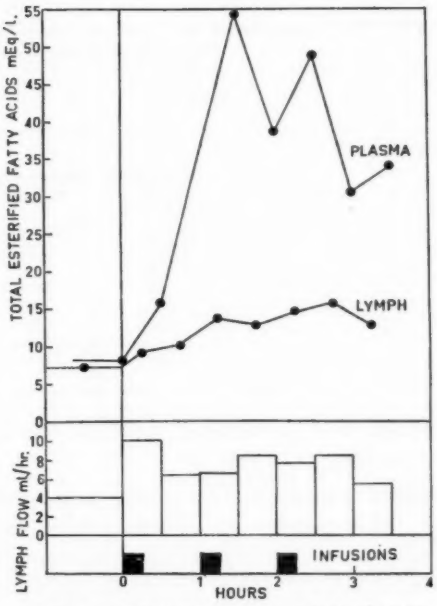

Fig. 1. Cat 3·2 kg. The effect of intravenous infusions of artificial fat emulsion on the total esterified fatty acids in plasma and hepatic lymph. At each of the times marked ■ 1 gm. per Kg. body weight of triolein in a 10 p.c. emulsion was infused.

Lipids in leg lymph before and after injury.

In the cat the plasma lipids are normally present mainly as α-lipoprotein with a high phospholipid:cholesterol ratio (Morris and Courtice, 1956). In a group of animals in which leg lymph was collected before and after injury, the concentrations of protein and of lipids in the lymph rose in relation to the plasma levels as follows: total protein from 50 to 69 p.c.; total cholesterol from 23 to 49 p.c. and phospholipid from 31 to 57 p.c. (Table 1). Although after scalding, the paw rapidly became grossly oedematous, the total protein concentration in the lymph did not reach as high a level relative to the plasma, as in the rabbit. This may be due to the high levels of the several globulin fractions in the cat. The increase in cholesterol and phospholipid in the lymph after scalding was asso-

TABLE 1.

The levels of total protein, cholesterol and phospholipid in the plasma and leg lymph of normal cats before and after injuring the paw by thermal burn.

No.	Plasma			Lymph					
	Protein gm. p.c.	Cholesterol mg. p.c.	Phospholipid mg. p.c.	Protein gm. p.c.		Cholesterol mg. p.c.		Phospholipid mg. p.c.	
				Before	After	Before	After	Before	After
8	7·8	347	308	3·0	5·3	52	179	62	171
9	6·6	103	200	3·3	4·7	27	42	80	120
10	8·0	64	144	3·5	5·6	14	34	49	88
11	8·8	112	125	4·7	5·8	46	52	65	72
12	—	109	215	—	—	30	57	53	108
	7·8	147	198	3·9	5·4	34	73	62	112

ciated with an increase mainly in the α-lipoprotein fraction as determined by zone electrophoresis.

Effect of infusion of fat emulsion on lipids in leg lymph.

Leg lymph was collected from the injured paw before and during the infusion of an artificial fat emulsion, homologous or autologous chyle, and heterologous (rabbit) hypercholesterolaemic plasma. In these experiments, even with damaged skin capillaries, there was very little transfer of the fat particles from plasma to lymph when infusions of artificial fat emulsion or chyle were made. With the dark-ground illumination there was in some cases an increase in the number of fat particles in the lymph after infusion. The amount of particulate fat in the leg lymph is normally very small, although some particles are always present; the number of particles may be increased without causing much measurable rise in the total fatty acid level which is present mainly in the α-lipoprotein. Fig. 2 depicts such an experiment.

The damage to the capillaries by scalding the paw increased the lymph lipids somewhat, but the subsequent intravenous infusion of 1·5 gm./Kg. body weight did not significantly change the lipid levels in the lymph although the concentration of particulate triglyceride in the plasma was raised to a high level. When hypercholesterolaemic plasma was infused, however, the cholesterol level in the lymph rose.

The passage of the lipoproteins in hypercholesterolaemic plasma across the injured capillaries is better shown in Fig. 3. In this experiment, the cholesterol and phospholipid levels in the lymph rose as the concentrations in the plasma increased. With dark-ground illumination and zone electrophoresis, these lipids appeared to be present as very fine particles or β-lipoprotein, but no measure of the amounts in each fraction were made. In this experiment the injured paw during three hours increased in weight by 52 gm. which is approximately one-third of the plasma volume.

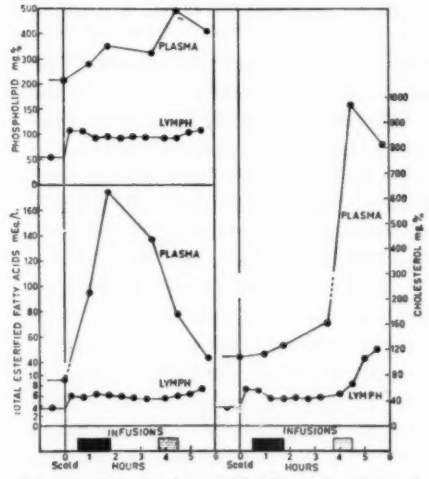

Fig. 2. Cat 3·0 kg. The effect of a thermal burn followed by intravenous infusions of an artificial fat emulsion, Lipomul, and of hypercholesterolaemic rabbit plasma on the lipid levels in the plasma and leg lymph.

▬ Lipomul, 1·5 gm. fat per kg.

⁞⁞⁞ Hypercholesterolaemic plasma.

Note that the scales for T.F.A. and cholesterol change, so that the changes in levels in the lymph can be better seen.

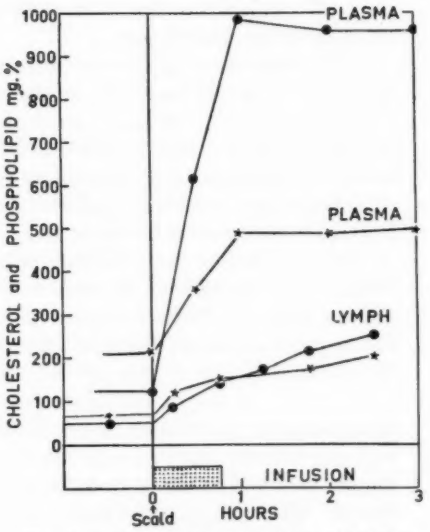

Fig. 3. Cat 3·2 kg. The effect of infusion of hypercholesterolaemic rabbit plasma on the levels of cholesterol and phospholipid in the plasma and leg lymph after a thermal burn to the paw.

● Cholesterol

★ Phospholipid

DISCUSSION.

The membrane of the hepatic sinusoids has long been known to be the most permeable of blood capillaries and this has recently been shown to be consistent with the structure of capillaries determined by the electron microscope (Bennett, Luft and Hampton, 1959). The capillaries of the skin on the other hand are very much less permeable to protein (c.f. Yoffey and Courtice, 1956) and structurally they contain no pores or fenestrations visible by electron microscopy. It has also been known that chylomicrons or particulate fat in the form of artificial emulsion, which on intravascular infusion rapidly disappear from the circulation, are largely taken up by the liver. The method of transfer of the particulate fat from the plasma to the liver cells has been controversial. The physiological rôle of intravascular hydrolysis by a lipoprotein lipase has not yet been clearly elucidated, but it is now believed that chylomicrons may leave the circulation without first being hydrolysed (c.f. Frederickson and Gordon, 1958). The present experiments show that when a high level of particulate fat in the plasma is maintained for three to four hours, the level in hepatic lymph

rises, although the actual amount of fat being returned to the blood stream in this way is less than 1 p.c.

It is difficult to get a clear understanding of the details of this process. The tissue fluid space between the hepatic sinusoidal membrane and the parenchymal cells is very small and has been doubted. The pictures which Bennett and his colleagues (1959) and Fawcett (1955) have obtained by electron microscopy, however, show that a definite space exists and that gaps up to several thousand Å between the endothelial cells of the sinusoidal membrane would allow fairly free transfer of molecules or particles up to this size. The lymphatics, however, are present only in the periphery of the liver lobule and any tissue fluid in the Dissé space would have to move to the periphery of the lobule before entering the lymphatics. Little is known of the pressures in this space, but the sinusoidal pressure would decrease from the periphery to the centre of the lobule. Tissue fluid in the centre of the lobule would, therefore, be moving from a region of lower to one of higher pressure and it would seem that if large gaps exist between the endothelial cells the tissue fluid might as readily pass back into the blood stream as into the lymphatics. The liver, however, has a high lymph flow and labelled proteins rapidly pass from the blood stream into the lymph. The present experiments show that fat particles up to 1 μ in diameter can also be transferred in this way. It is not known, however, whether the lymph is derived from all parts of the liver lobule, or whether it is derived mainly from the tissues in the periphery of the lobule where the lymphatic capillaries are situated. Whichever is true, it seems probable that the fat particles in the lymph after an infusion of fat emulsions have passed through the large gaps in the sinusoidal membrane without first being hydrolysed.

Through the capillaries of the skin of the hind paw, where there are no gaps in the endothelium, the plasma lipoproteins are normally exchanged, but the plasma:lymph gradient is slightly greater than in the rabbit. As the size of the lipoprotein molecules in the rabbit and cat have not been determined, it is not possible to say whether the molecules are larger in the cat or the permeability of the capillary membrane is less.

Damage to the capillary membrane by heat increased the permeability to

TABLE 2.

The levels of total protein, cholesterol and phospholipid in lymph from the liver and from the skin of the normal and scalded paws of cats relative to those in the plasma.

	Plasma	Hepatic Lymph	Lymph from leg	
			Normal	Injured
Total Protein	100	89	50	69
Cholesterol	100	84	23	49
Phospholipid	100	88	31	57

proteins and lipoproteins, but the plasma:lymph gradient was still greater than that observed in the liver by Morris (1956). The comparable figures are given in Table 2. The membrane of the damaged skin capillary in which the flow and pressure of blood is high seems to be less permeable than the normal hepatic sinusoids to protein and lipoproteins.

The larger lipid particles of chyle or of artificial fat emulsions, 0·5-1·5 in diameter, which pass through the hepatic sinusoids do not penetrate the membrane of the skin capillaries except in small numbers even when the capillaries are damaged by heat. In this preparation it is probable that if particulate fat were transferred across the capillary membrane it would enter the lymph, because of the rapid formation of gross oedema with increase in lymph flow. In such conditions the tissue fluid and lymph are probably of approximately the same composition as capillary filtrate. The relatively small increase in the level of fat particles in the lymph suggests that they are not readily transferred across the walls of the skin capillaries.

When hypercholesterolaemic rabbit plasma was infused, however, there was a rapid transfer of the lipoproteins across the capillary membrane. These lipoproteins are foreign (but the capillary membrane was already damaged by heat), much smaller in size and of different lipid composition as compared with the artificial fat emulsion and chyle. It is tempting to suggest, as in the rabbit, that size of the lipoproteins is at least one of the factors governing their transport across these membranes. If the endothelium of the aorta and large arteries behaves in the same way as that of the skin capillaries, it would seem that the larger chylomicrons would not easily be transferred, but that the smaller, low density lipoproteins would penetrate the endothelium.

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THE EFFECT OF SALICYLATE ON PLASMA PROTEIN BOUND IODINE IN MAN[1]

by JOHN S. CHARNOCK,[2] BRIAN F. GOOD[3] AND BASIL S. HETZEL[4]

(From the Department of Medicine, University of Adelaide, Adelaide, South Australia).

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SUMMARY.

The effect of a large single oral dose (5 gm.) of sodium salicylate on plasma PBI has been compared with that of a similar dose of sodium parahydroxybenzoate.

Although a significant fall in plasma PBI occurred after two hours following both agents, the fall with salicylate was significantly greater ($P < 0{\cdot}05$).

A similar fall in plasma PBI was observed in a diabetic patient given salicylate in a dosage of 60-100 grains per day with a plasma salicylate level of 21-31 mg. p.c.

The possible mechanisms involved are discussed and it is concluded that the effect may be due to an action on the feed back mechanism for thyroid hormone in the hypothalamus.

INTRODUCTION.

A study of the acute metabolic effects of a single dose of 5 gm. sodium salicylate in man has recently been reported from this laboratory (Hetzel *et al.*, 1959). Certain effects on electrolyte excretion were demonstrated similar to those following hydrocortisone. However, hydrocortisone could not account for the marked elevation of metabolic rate and fall in plasma cholesterol which were observed (Hetzel, *et al.*, 1957). These findings raised the possibility of thyroid activation. However, this seemed unlikely in view of the observation of similar changes in myxedema patients given salicylate (Alexander and Johnson, 1956). Nevertheless, an examination of thyroid function following salicylate was thought desirable. This was carried out by a study of the plasma protein-bound-iodine (PBI) as an indication of the level of circulating thyroid hormone in the experiment previously reported. In addition to these acute observations, one study following the chronic administration of salicylate to

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[2] Supported by a whole time grant from the National Health and Medical Research Council of Australia.

[3] Research Assistant, Michell Foundation, University of Adelaide.

[4] Michell Reader in Experimental Medicine, University of Adelaide.

man has also been carried out. Since these observations were completed the report of Austen *et al.* (1958) describing studies of thyroid function following the chronic administration of salicylate to man has appeared.

METHODS.

Acute studies.

Five gm.[5] of sodium salicylate were given orally to six healthy young male medical student volunteers. A similar dose of parahydroxybenzoic acid was also given to these students as a control.

The drugs were given seven days apart and their administration was randomised to minimize environmental factors. Each drug was given in solution, suitably flavoured with aniseed.

Oxygen consumption was determined (using the Jones machine as previously described —Hetzel *et al.*, 1959) during a one-hour preliminary control period on both days, and urine and blood samples were collected. The drug was then taken and further samples of urine and blood were collected at hourly intervals for four hours. Oxygen consumption was determined again approximately 90 and 210 minutes after ingestion of the drug.

Chronic study.

The patient (G.E.) was a female diabetic aged 52. She presented with polydipsia, polyuria and glycosuria. She had extensive xanthomata on the arms and plasma cholesterol was 388 mg. p.c. A glucose tolerance test confirmed diabetes mellitus.

On admission, the patient was placed on an 1800 Calorie unsweetened ward diet. After a five-day baseline period salicylate in the form of calcium acetyl-salicylate was administered in a dosage of 100 grains a day. This was reduced to 60 grains a day after four days owing to the severity of tinnitus and was continued for a further eleven days.

Serial determinations of plasma PBI, plasma cholesterol, plasma salicylate, and haematocrit were carried out over the period of salicylate administration.

Plasma salicylate was determined by the method of Trinder (1954) and plasma cholesterol by a modification of the method of Zak *et al.* (1954). Plasma protein-bound-iodine was determined by the alkaline ashing technique of Acland (1957) after a thorough investigation of the method. Each step in the procedure was critically examined and rigid control was observed throughout. All estimations were performed at least in duplicate, control analyses on a pooled plasma sample were included with every batch and occasional recoveries of added thyroxine were included throughout the period of estimations. Direct addition of either salicylate or parahydroxybenzoate to plasma had no effect on the chemical determination of PBI as carried out in this laboratory.

Statistical analysis of the PBI data showed that there was a S.E. between batches of 0·177 μg. p.c. and a S.E. of the mean of two duplicates of 0·125 μg. p.c. Hence a variation of greater than $\pm 0{\cdot}25$ μg. p.c. was of statistical significance ($P < 0{\cdot}05$) under these conditions. A full discussion of the method will be presented elsewhere.

RESULTS.

Acute studies.

Plasma salicylate. Values have been previously reported (Hetzel *et al.*, 1959). After the first hour levels varying between 24 and 40·5 mg. p.c. with a mean of 33·1 mg. p.c. were obtained. There was a further slight rise after

[5] One student weighing 97 kilos was given 7 gm. of each salt.

two hours – the mean level being 38·1 mg. p.c. (range 30·5-42·5). The mean level was 34·6 mg. p.c. (range 26-45) after three hours and 32·1 mg. p.c. (range 28-38·5) after four hours.

TABLE 1.

Effect of sodium salicylate (S) on plasma PBI μg. p.c. compared with sodium parahydroxybenzoate (P).

Subject	Treatment	Time in Hours				
		0	1	2	3	4
A	S	4·0	4·9	4·6	5·4	4·4
	P	4·6	4·6	4·5	4·1	4·5
B	S	5·1	4·3	3·2	4·0	6·6
	P	4·4	4·4	4·0	4·4	3·9
C	S	6·2	4·9	4·5	5·5	6·0
	P	6·3	5·7	5·7	5·0	6·1
D	S	4·4	4·2	3·5	3·0	3·5
	P	5·0	4·5	4·2	4·3	4·9
E	S	5·3	4·7	4·9	4·2	4·0
	P	6·5	5·7	5·4	5·7	5·9
F	S	5·4	4·2	4·7	5·2	4·7
	P	5·8	4·6	5·9	4·2	5·7
Mean	S	5·1	4·5	4·2	4·6	4·9
	P	5·4	4·9	5·0	4·6	5·2
P*		ns	ns	<0·05	ns	ns

P* probability using "t" test. ns=not significant.

Plasma PBI—values are given in Table 1. The initial levels are well within the normal range (3-8 μg. p.c.) There was a fall in plasma PBI following sodium parahydroxybenzoate after 2 hours ($P < 0·05$). Comparison of the levels at this time revealed a further significant fall ($P < 0·05$) following salicylate.

Oxygen consumption. Mean increases of the order of 38 p.c. were apparent by 90 minutes in the subjects who received salicylate and this increase was still evident after a further two hours (Hetzel *et al.*, 1959). No increase was observed following sodium parahydroxybenzoate.

Chronic study.

The effects on plasma salicylate, PBI and cholesterol are summarized in Table 2 (Fig. 1).

It will be seen that the plasma salicylate rose to a maximum level of 31 mg. p.c. while the plasma PBI fell to 3·6 μg. p.c. from initial values of 6·1 μg. p.c.

TABLE 2.

Effect of chronic administration of salicylate on plasma PBI and cholesterol.
(Case of diabetes mellitus with hypercholesterolaemia)

Day	Plasma salicylate mg. %	Plasma PBI μg. %	Plasma cholesterol mg. %
−3	3·5	6·0	388
−1	2·0	5·8	325
+1	17·0	5·1	318
+3	31·0	4·3	280
+6	27·0	3·6	285
+12	27·0	3·7	290
+14	21·0	4·3	288

and 5·8 μg. p.c. before salicylate administration. There was an associated fall in plasma cholesterol from 388 mg. p.c. and 325 mg. p.c. before to 280 mg. p.c. after salicylate.

DISCUSSION.

Studies of the acute effects of a large, single dose of sodium salicylate on plasma PBI have not previously been reported. The results indicate a significant fall in two hours in association with plasma salicylate in excess of 30 mg. p.c.

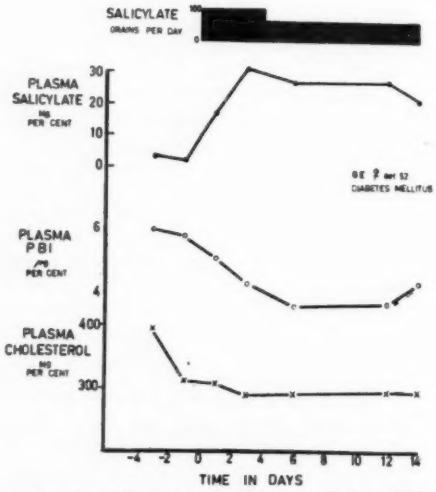

Fig. 1. Effect of salicylate on plasma PBI and plasma cholesterol in a diabetic patient with hypercholesterolaemia.

In the chronic study the fall in plasma PBI in the diabetic patient was similar to that previously reported by Austen *et al.* (1958). A fall in plasma PBI has also been demonstrated with chronic administration of salicylate to rats in this laboratory (Charnock, 1958, unpublished). Austen *et al.* (1958) were also able to show a 45 p.c. reduction in the uptake of radioiodine after two hours with no significant change in renal clearance following salicylate.

A number of suggestions may be made as to the mechanisms involved in this fall in plasma PBI. A direct effect on the thyroid has been excluded by the experimental studies of Austen *et al.* (1958) by observations on thyroid slices and the rat thyroid blocked with thiouracil.

The suggestive evidence of adrenal cortical stimulation by salicylate in guinea pigs and rats (Good *et al.*, 1957; Hetzel and Hine, 1951) raises the possibility that the fall in PBI is the result of hydrocortisone secretion. The sodium retention and potassium loss which occurred simultaneously with the fall in plasma PBI in this experiment suggest adrenal cortical activation (Hetzel *et al.*, 1959). Brown-Grant (1955) has shown an inhibition of the release of thyroid radioiodine in the rat by cortisone. Hills *et al.* (1950) found that ACTH and cortisone depressed the plasma PBI and uptake of radioiodine in man. This suppression could be overcome by the administration of exogenous TSH as has also been shown by Wolff and Austen (1958) following salicylate. However, a recent study using C^{14} (Peterson *et al.*, 1958) has failed to demonstrate a significant increase in the plasma concentration or rate of metabolism of hydrocortisone following salicylate in man as previously suggested (Done *et al.*, 1958; Hetzel *et al.*, 1959.)

The similar fall in PBI resulting from the administration of salicylate, dinitrophenol and other agents which increase oxygen consumption such as pyrogens (Castor and Beierwaltes, 1956; Goldberg *et al.*, 1957) has suggested that the fall is related to this increase in metabolic rate. The rate of disappearance of injected radiothyroxine from the circulation has been shown to be increased during chronic salicylate administration and this increase has been correlated with the rise in oxygen consumption (Austen, *et al.*, 1958). A normal PBI has, however, been found in other states of hypermetabolism with a similar increase in disappearance rate of radiothyroxine (Sterling and Chodos, 1956).

Studies of mitochondrial preparations have shown that there is an uncoupling of oxidative phosphorylation by salicylate and dinitrophenol (Brody, 1956; Charnock, 1957, unpublished). However, it has been possible to dissociate these two effects in the case of the dihydroxybenzoic acids which do not uncouple oxidative phosphorylation (Brody, 1956) though they do depress release of I^{131} from the rat thyroid (Wolff and Austen, 1958).

Another alternative is that the fall in PBI is due to action at the hypothalamic level. The intensive studies of Goldberg *et al.* (1955) on the fall in PBI following dinitrophenol may be relevant. There appears to be an inhibition of TSH production associated with "inactive" pituitary cytology under these conditions. This effect has recently been discussed by Brown-Grant (1957). Perhaps both salicylate and dinitrophenol produce a specific effect—decreasing the normal sensitivity of the feed back mechanism regulating TSH activity in relation to plasma PBI. This mechanism is probably activated from the hypothalamus where a specific centre controlling thyroid function has been demonstrated in dogs and rats (Ganong *et al.*, 1955; D'Angelo, 1958). The interference by salicylate with goitre formation in thiouracil-treated rats strongly suggests action at this site (Wolff and Austen, 1958). A site of action of salicylate in the hypothalamus has also been suggested to explain the abolition

of the effect of salicylate on adrenal ascorbic acid by "dial" anaesthesia in rats (Hetzel and Hine, 1951; Van Cauwenberge and Betz, 1952). Such a mechanism does not appear to be related to the antipyretic action of salicylate, as this appears to depend on the peripheral metabolic stimulating effect (Reid, 1957).

A fall in plasma PBI has also been described in rats following severe stress (Van Middlesworth, 1951; Williams *et al.*, 1949) and cold exposure (Bondy and Hagewood, 1952) though there is good evidence of thyroid activation with the latter (Stevens *et al.*, 1955). However, under other conditions in man elevation of PBI may occur (Board *et al.*, 1956; Hetzel *et al.*, 1956; Schwartz and Roberts, 1957). It seems likely that these stimuli are also acting at the hypothalamic level, though it is apparent that the actual level of plasma PBI is influenced by both central and peripheral factors.

The fall in plasma cholesterol noted in the hypercholesterolaemic diabetic patient is similar to that previously described in myxedema (Alexander and Johnson, 1956). A fall in plasma cholesterol was also noted in the acute experiment in the normal subjects and has previously been reported (Hetzel *et al.*, 1959). It has since been confirmed using a more specific method (that of Zak *et al.*, 1954). It seems likely that this change is closely related to the peripheral metabolic stimulant effect of salicylate (Alexander and Johnson, 1956; Hetzel *et al.*, 1959).

The fall in plasma PBI following sodium parahydroxybenzoate may be related to the interference with thyroid hormone biosynthesis which has been demonstrated in thyroid slices with this agent (Taurog *et al.*, 1945). There was no consistent change in the haematocrit following either agent, so the fall was not due to increase in plasma volume.

In subsequent studies (Hetzel, *et al.*, to be published) the effect of salicylate on PBI in various states of thyroid function has been examined. Striking falls have been noted in eight cases of hyperthyroidism but only minimal changes have been observed in four hypothyroid patients maintained on desiccated thyroid. This latter finding supports the possibility of a central effect of salicylate on thyroid function.

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THE HAEMATOLOGY OF THE CENTRAL AUSTRALIAN ABORIGINE[1]

11. WHITE AND DIFFERENTIAL COUNTS; EOSINOPHIL COUNTS AND CASONI TESTS

by J. R. CASLEY-SMITH

(From the Department of Anatomy, University of Adelaide).

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SUMMARY.

Total leukocyte and differential counts were performed on a group of aborigines. These lived under semi-detribalized conditions at Haast's Bluff. A control series was taken of Europeans living at Adelaide.

The total leukocyte and absolute lymphocyte counts were slightly but significantly raised in the aborigines. The eosinophil counts were markedly increased in the aborigines.

Direct eosinophil counts were performed on nomads visiting Haast's Bluff. These also had greatly raised values. Many of them had positive Casoni tests. Those with positive tests had more eosinophilia than those with negative reactions. Hospital natives had fewer positive reactions.

INTRODUCTION.

Part I and others of this series of papers set forth the haemoglobin levels, and a number of other features of the red cells of Central Australian aborigines. Since the white cells in these people have only been partly studied (Maegraith, 1938), it seemed desirable to record various other data about them.

A group of semi-detribalized full aborigines was studied in 1956. They were living at Haast's Bluff Settlement, 160 miles west of Alice Springs. They were compared with a control series of Europeans living at Adelaide. In addition, some direct eosinophil counts were performed on a nomadic group of Pintubis, who came to Haast's Bluff in 1957. Many had not seen white men before. Other eosinophil counts were done on natives at the Alice Springs Hospital. They were under treatment and had come from various centres. Casoni tests for hydatids were carried out on these last two groups.

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[1] The data upon which this work is based were collected during two University of Adelaide Anthropological Expeditions. These were financed by grants from the Wenner-Gren Foundation for Anthropological Research, New York, and the Medical Research Fund of the University of Adelaide.

Materials and Methods.

The semi-detribalized natives had lived round the Settlement for 15 years or more. Differential counts were performed on about equal numbers of both sexes. Unfortunately, there was difficulty in obtaining the necessary diluting fluid for the total white counts because of transportation in the field, hence far fewer males were examined in this way. However, most workers do not find a significant difference between European males and females so in those results dependent upon the total white count, the aboriginal male and female figures were combined.

The control series of Europeans was obtained from donors at the Red Cross Blood-Taking Centre, Adelaide, and from students. These were matched as closely as possible for age and sex with the aborigines.

Blood was taken with the minimum possible application of a tourniquet (M.R.C., 1943). Films were made at once and stained by Leishman's method. One hundred cells were counted: the numbers made this total adequate. Unfortunately, conditions in the field precluded doing the total white counts at once. The blood was diluted and the resulting fluid put in dry, air-tight bottles. These stood for four to six hours. The fluid was well mixed before counting. Inevitably some cells would be lost by adsorption and lysis, but this would apply equally to all groups, male and female, aboriginal and European. Hence the results are comparable between groups but not with those of other workers. The counting apparatus was checked and the methods standardized as far as possible. On the 1957 expedition a few more white counts were done on males who lived at the Settlement.

The eosinophils were estimated directly using an eosin stain as a diluent. As this method is easily standardized a control series was not done, but the results are compared with those obtained by others. Casoni skin tests were done using a saline control. The serum was obtained from the Commonwealth Serum Laboratories. The group of hospital patients and their visitors were examined in a similar manner.

RESULTS.

Throughout this paper the following symbols indicate the relevant significance levels:—

x = just significant	$\cdot 05 < P < \cdot 01$
xx = significant	$\cdot 01 < P < \cdot 001$
xxx = very significant	$\cdot 001 < P$.

Figures in brackets following a result are the Standard Errors. Tests were "t tests" unless Behren's tests (Fisher and Yates, 1953) were indicated.

Total leukocyte counts.

The 16 aboriginal males had a mean of 6565·6 (551·9) cells/cmm., and a mean age 24·09 years. The 52 females' mean was 6810·6 (293·6), and their mean age 26·54. The 29 European males had 5617·2 (437·1) with a mean age of 36·07; the 74 females' mean was 5654·1 (191·6) with mean age of 30·07. As the variances of the four groups did not differ significantly among themselves, they were combined to give a grand variance of 3,999,350 giving a S.D. of 2000·0. There were no significant differences between the males and females in either ethnic group. So combination was possible. This gave 68 aborigines with a mean of 6753·0 (242·5) and 103 Europeans with one of 5643·7 (197·1).

The difference between these was very significant (xxx). There was no significant difference between the two groups of males, but there was between the females (xx).

Differential counts—percentages.

1. *Neutrophils.* The 42 aboriginal males had a mean of 60·333 p.c. (1·658), while the 60 females had one of 56·083 (1·388). The 62 European males had 61·371 (1·364) and the 76 females 60·250 (1·232). The variances were not significantly different. Combined they gave a grand variance of 115·435 and S.D. of 10·744. There were no significant differences between the sexes in either of the ethnic groups. The 102 aborigines had a mean of 57·833 (1·063) and the 138 Europeans had 60·754 (0·915). There was a just significant difference (x) between these values. The difference between the two groups of males was not significant, but that between the females was (x).

2. *Eosinophils.* The distributions were markedly skew, so the square roots of the percentages were used to make them more "normal". Eventually these were converted back for presentation. Significance tests were, of course, done on the square roots. The aboriginal males' mean was 5.233 p.c. (0·568), the females' was 8·450 p.c. (0·928). The Europeans had 2·083 (0·295) and 2·260 (0·277) respectively. The variances of aboriginal males and both European groups could be combined to give a grand variance of 0·4150 and S.D. of 0·6444 p.c. There was a significant (xx) difference between the sexes in the aborigines, but not in Europeans. These latter were combined to give a mean of 2.180 (0·202). Both aboriginal groups were very significantly different from the European.

3. *Basophils.* These cells occurred so infrequently that the results, especially the S.E.'s, mean very little. The aboriginal males had a mean of 0·50 (0·014), the females one of 0·38 (0·073). The Europeans had 0·35 (0·074) and 0·26 (0·051) respectively. Combining gave 0·43 (0·06) for the aborigines and 0·30 (0·044) for the Europeans. The results were tested by comparing the number of slides with none, or one or more, cells in each group. These dichotomies were tested by the χ^2 test. No difference was significant between any group, nor between the combined groups.

4. *Lymphocytes.* The aboriginal males' and females' means were 28·000 p.c. (1·458) and 29·183 (1·221) respectively. The Europeans' were 31·000 (1·201) and 31·553 (1·196). The variances were not significantly different. Combining them gave a grand variance of 89·323 with S.D. of 9·451. There were no significant sex differences in either ethnic group. The combined Aborigines' mean was 28·696 (0·936) and the Europeans' 31·304 (0·805). The difference between these means was just significant (x). The difference between the two male groups was not significant, nor was that between the two groups of females.

5. *Monocytes.* Again the data were skewed and square roots had to be

used. The mean of the aboriginal males was 5·105 (0·259) and that of the females was 4·061 (0·157). The means of the Europeans were 4·475 (0·253) and 4·449 (0·310). There was a very significant (xxx) difference between the sexes in the aborigines, but not in the Europeans. The combined results of the latter gave a mean of 4·460 (0·200). This was just significantly (x) less than the aboriginal males but not significantly greater than the females.

Differential counts—absolute values.

These were obtained by multiplying the percentages obtained from the films by the total leukocyte count for that film. Hence they also should not be compared with results obtained by other workers, but may be compared among themselves. There were only five aboriginal males represented, so, unless otherwise mentioned, they were combined with the women to give a "total aborigines group".

1. *Neutrophils.* The 53 aborigines had a mean of 3859 cells/cmm. (214), the 23 European males had one of 3330 (220) and the 68 females 3495 (154). The Europeans did not differ significantly between the sexes; their combined mean was 3454 (127). There were no significant differences between the combined aboriginal group and the combined European one, nor between the two groups of females.

2. *Eosinophils.* Again the square roots were used in the calculations. The aborigines' mean was 562·5 (70·0). There was no significant sex difference. The European males' mean was 150·5 (31·7), the females' was 141·5 (20·3). These two were not significantly different. Combined, their mean was 143·5 (17·1). This was very significantly (xxx) less than the aboriginal group.

3. *Basophils.* Again quantity of cells per film make the results of little meaning. The aborigines had a mean of 74·1 (10·6). Those of the European males and females were 72·8 (16·6) and 53·7 (6·2) respectively. Combining the Europeans gave a mean of 58·5 (6·2). Again the films were divided into dichotomies, those with and those without 50 or more cells/cmm.: χ^2 tests revealed no significant differences between ethnic groups or sexes.

4. *Lymphocytes.* The aborigines' mean was 1950 (113·5), while the European males' was 1485 (101·1) and females' was 1759 (78·5). There was no significant sex difference in the Europeans; their combined mean was 1689 (65·1). This was just significantly (x) less than the aboriginal mean.

5. *Monocytes.* Once more square roots were used in the calculations. The aborigines had a mean of 285·8 (25·1). The Europeans had 257·3 (36·2) and 259·7 (21·1) respectively for men and women. The combined Europeans had 259·2 (18·3) as their mean. There were no significant differences between variances, so combining them gave a grand variance of 58·5477 and a S.D. of 7·652. Except for the basophils, this was the only type of cell where the aborigines' variance was not significantly greater than that of the Europeans. There were no significant differences between ethnic groups, or sexes.

Direct eosinophil counts and Casoni tests.

The 36 nomadic Pintubis had a mean of 884·5 cells/cmm. (98·1). The seven hospital natives' mean was 650·6 (161·8). In the nomads, males vastly predominated. The two means were not significantly different. Of the Pintubis 23 Casoni tests revealed nine positives and 14 negatives. The positive ones' mean was 1362 cells/cmm. (224·0); that of the negatives was 770·2 (131·2). These were just significantly different (x). As the distributions of these values were skewed, the effect was tried of taking the square roots and the square roots of the logarithms of the original values. The results were very similar, though the distributions looked more "normal". These results may be contrasted with the mean value of 200/cmm. given by Wintrobe (1956). The mixed group of hospital natives had 19 negative and two positive Casoni reactions. These people were drawn from divers places; many had been living on stations under near-European conditions.

DISCUSSION.

The total leukocyte counts were higher in aborigines than in Europeans. While all types of cells showed an increase in their absolute values, only in the eosinophils and lymphocytes was this increase significant. The percentages obtained from the films are mutually dependent, so it is best to consider only the absolute values of each type of cell. As most published series show no sex differences the preponderance of females in the aboriginal series is unlikely to affect the results.

There are many factors which may influence the leukocyte count. Emotion, exertion and exposure, especially to short electro-magnetic radiations, cause a lymphocytosis—both relative and absolute (Clark, 1921; Clark, 1922; Stammers, 1933; Peterson and Peterson, 1935; Sturgis and Bethell, 1943). It appears that both a reduction in atmospheric pressure and an increase in ultraviolet irradiation cause a lymphocytosis. While exertion may also do this, one could not describe these settlement natives as particularly energetic. In addition, mild, regular exertion as opposed to sudden bursts of activity does not alter the leukocyte picture (Bookman and Fraad, 1935). The high iron diet of the aborigines (Wilson, 1953; Dadswell, 1934) may also contribute to the leukocytosis (Sturgis and Bethell, 1943). Wintrobe (1956) says that leukocytosis due to these factors may be distinguished from that due to infection by an absence of a shift to the left in the Arneth count. However, this would surely apply only to a brief exposure. It would seem that prolonged exposure as occurs in the aborigines may well cause a leukocytosis with more young cells present. Maegraith (1938) and Casley-Smith (1959) have recorded a marked increase in young leukocytes in aborigines. This has also been observed in other exposed native peoples by other workers.

It is of interest to note that the aborigine, again in company with other native peoples, shows an increase in a_2 and γ globulins (Wilkinson *et al.*, 1958), the so-called "stress pattern". This may have a relation to the observed lymphocytosis – also a "stress pattern". Another possibility is the greater susceptibility of the aborigine to infections. This is due to his rigorous environment and contact with European diseases. Possibly both factors play a part.

Eosinophilia.

It is almost commonplace to discover extensive eosinophilia in native people living under primitive conditions. This is due to their many parasitic infestations, skin lesions and other diseases. Davis and Pitney (1957) found considerable relative eosinophilia among aborigines in the Warburton Ranges. Horsfall (1958) saw some in aborigines around Cairns – here usually due to hookworm. Nevertheless, in Central Australia the environment is most unsuitable for hookworm. The natives were all apparently healthy with no suggestion of "tropical eosinophilia" as described by Weingarten (1943). However, scabies is very common and probably played a part.

Hydatids is very common in Australia generally, though not searched for in this area before. The close contact with dogs, cattle and kangaroos and lack of cleanliness make this disease quite likely. Complement fixation tests were performed (Warner *et al.*, 1957). Unfortunately, the only suitable sera available happened to come from individuals with low eosinophil counts. The results were negative. However, the large number (40 p.c.) of positive Casoni reactions shows that the possibility of chydatid infestation should not be ignored especially as the positives were associated with increased eosinophil counts. On the other hand, it is unlikely to be the only cause. Individuals with negative Casoni reactions still had raised eosinophil counts. It should also be realized that the Casoni test is not completely reliable, and that "false" positives are possible with other helminthic infections. The smaller number of positives (10 p.c.) in the hospital natives is probably due to their divers origins and generally better living conditions than amongst the Pintubis.

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A STUDY OF THE METASTATIC DISTRIBUTION OF ERLICH ASCITES TUMOUR CELLS IN MICE[1]

by E. E. SELECKI

(From the Unit of Clinical Investigation, Royal North Shore Hospital, and the New South Wales Red Cross Blood Transfusion Service, Sydney).

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SUMMARY.

The introduction of a method of labelling ascites tumour cells with radioactive chromium has facilitated a quantitative study of metastasis of these cells.

A rapid rate of removal of tumour cells from the blood stream takes place in the first 15 minutes after the intravenous injection of labelled tumour cells, and a corresponding rise in the number of tumour cells lodged in the lungs is observed at this time.

A constant release of tumour cells from the lungs into the systemic circulation occurs immediately after the intravenous injection. The release is relatively slight in the first three hours, but after 24 hours only 5 p.c. of the number of cells accumulated remain in the lung.

The liver accumulates about 20 p.c. of the total number of cells injected in the first six hours. The subsequent disappearance of the cells from the organ is slow.

A steady accumulation of cells is observed in the spleen up to the third day after injection. The total number of cells reaching the spleen is small, but the concentration per gram of splenic tissue is relatively high.

INTRODUCTION.

The majority of deaths occurring from neoplastic disorders is due to metastatic infiltration of vital organs in the body. Clearly, therefore, the study of metastasis is of great importance. In the past such studies have mostly been devoted to the macroscopic and microscopic characters of metastatic tumours. The present work aimed at developing a quantitative method of observing the distribution and fate of injected tumour cells. It was hoped thereby to throw light on the early stage of the metastatic process, namely, on the initial phase of transport and distribution of the detached tumour cells. For our purpose, ascites tumour cells offered advantages:—

(i) A known number of tumour cells could be injected.

(ii) To obtain cell suspensions, no disintegrating mechanical procedures (such as are required to obtain cell suspensions from solid neoplasms) were necessary.

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(iii) No large aggregates of cells and no interstitial tissue were injected.

Warren and Gates (1936) were the first to use ascites tumours in the study of metastases.

Little information is available at present concerning the early stages of dissemination of tumour cell emboli. Coman, DeLong and McCutcheon (1951) injected stained and fixed tumour cells into the left heart of rabbits and studied their distribution in histological sections. Their results with the tumour and species studied were in accord with the "mechanical" hypothesis of metastasis, the conclusion being that the distribution of embolic tumour cells is adequately accounted for by the mechanics of the circulation, and that the development of metastasis depends mainly on the number of tumour emboli reaching a given capillary bed. The rôle played by local chemical ("soil") factors is, in the opinion of the authors, a minor one if it exists at all.

Goldie, Jeffries, Jones and Walker (1953) injected blood and mashed organs suspected of containing metastatic growth intraperitoneally into mice. The rate of growth of the resulting ascites tumour was used as a semi-quantitative measure of the number of metastatic cells present in the organ. Again the results support the "mechanical" hypothesis for the location of metastases, which implies that this depends primarily on the numbers of tumour cell emboli and the anatomical arrangement of the vascular system.

First attempts to label ascites tumour cells with radioactive phosphorus and quinacrine were made by Ambrus, Ambrus, Byron, Goldberg and Harrisson (1956). The obvious disadvantage of P^{32} labelling lies in the fact that there is a comparatively free exchange of P^{32} between the labelled, washed tumour cells and the tissues of the host. The exchange is a source of error and makes the method applicable only to short-term experiments.

In 1958 Rajam, Jackson and Black described the use of radioactive sodium chromate for labelling ascites tumour cells. Their method was analogous to that used by Gray and Sterling (1950) for labelling erythrocytes. According to Rajam, the label obtained was largely intracellular, the precise location of the chromium within the cell, however, being unknown. The elution of radioactivity from the cells was slightly more than 1 p.c. per day *in vitro* and was not appreciable unless cell rupture occurred (Rajam *et al.*, 1958).

In the experiments reported here attempts were made to label ascites tumour cells by Rajam's method, and after injecting them intravenously into mice, to follow their fate and distribution by measuring the radioactivity of various organs at different time intervals.

Materials and Methods.

Labelling of tumour cells.

Ascites fluid containing tumour cells was withdrawn from the peritoneal cavity of Swiss mice and placed in a tube containing physiological saline. This heavy suspension

was then diluted to a concentration of 3×10^6 cells per ml. 30 ml. of this solution was placed in a stoppered tube and 90 μc of $Na_2Cr^{51}O_4$ were added, giving a concentration of 10 μc to 10^7 cells. The cells were incubated at room temperature by rotating the tube at 15 r.p.m. for 1 hour.

After incubation, 100 mg. of ascorbic acid was added. As shown by Read (1954) the addition of ascorbic acid, by reducing the excess of tetravalent chromium to the trivalent form, prevents the uptake of Cr^{51} by red cells. Thus the radioactivity of a sample of packed red cells from an injected animal would correspond only to the radioactivity of the biologically intact tumour cells in the blood.

One ml. of the suspension was taken and the total amount of radioactivity determined by counting in a well-type scintillation counter. The solution was then spun down at 800 r.p.m. for four minutes and the radioactivity of 1 ml. of the supernatant was determined. After five consecutive washings, each with 30 ml. of saline, the radioactivity of the supernatant fluid reached constant "plateau" values.

The experiment was then repeated in full with lesser amounts of radioactivity (5 μc to 10^7 cells).

Viability of labelled cells.

The viability of labelled cells was determined by testing their ability to form solid subcutaneous tumours in mice. Aliquots (0·5 ml.) taken from the washed cell suspension labelled with 10 μc of $Na_2Cr^{51}O_4$ to 10^7 cells, and similar 0·5 ml. aliquots from the suspension labelled with 5 μc. of $Na_2Cr^{51}O_4$, were injected into the loose subcutaneous tissue on the necks of 12 mice.

Intravenous injection of labelled cells.

A washed cell suspension of 30 ml. containing 9×10^7 cells was labelled with 90 μc of Cr^{51}. It was then divided into 0·5 ml. aliquots each containing $1{\cdot}5 \times 10^6$ cells and these aliquots were injected intravenously into mice. The radioactivity of two aliquots was counted and these samples were kept as standards to determine radioactivity decay during the course of the experiment. The weight of the animals used ranged from 20 to 24 gm.

Before injection, the jugular vein of the animal was exposed as it emerged at the cranial part of the pectoral muscles. Pressure was then applied to the pectoral muscle overlying the vein and a 28-gauge needle attached to a fine syringe was inserted into the distended vein through the muscle. The pressure was then removed and after completing the injection the needle was withdrawn through the pectoral muscle. No haemorrhage occurred.

No animal died after the injection of $1{\cdot}5 \times 10^6$ cells in 0·5 ml. suspension. When the dose was increased to 1 ml. and 3×10^6 cells, some animals died with signs of acute respiratory distress. Autopsy revealed pulmonary oedema and, in some, gross pulmonary infarcts.

Determination of radioactivity of various organs and of the blood.

At increasing time intervals after the injection (5 minutes, 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours, 3 days, 1 week) the animals were exsanguinated and sacrificed. A few drops of heparin were injected intravenously into each animal before bleeding. It was then possible to collect up to 1 ml. of venous blood from the jugular vein for examination.

The lungs, liver, kidneys and spleen were removed, weighed and homogenised with a rotating type homogeniser. The aqueous portions were retained and the total radioactivity of the whole organ and the radioactivity per gram of organ determined. From the specific

activity of the individual tumour cells injected, the number of intact tumour cells in the whole organ and the concentration per gm. of organ could be deduced.

From the volume of packed red cells in the blood sample and its total radioactivity, the radioactivity per ml. of packed red cells was determined and the corresponding number of tumour cells deduced.

The blood volume of each experimental animal was calculated from the formula of Oakley and Warrack (1940). A haematocrit reading, corrected for trapped plasma, was made and the total volume of packed red cells in the animal was calculated. From the number of tumour cells per ml. and the total volume of packed red cells, the number of tumour cells in the whole of the blood stream was calculated.

Attempts were made to determine the amounts of residual blood in the organs after their removal, since the radioactivity eluted from ruptured and destroyed cells may be taken up by the serum protein and constitute some source of error. The aqueous samples containing the homogenised organs were centrifuged at 3000 r.p.m. for 15 minutes and after complete sedimentation of the particulate matter, attempts were made to determine the haemoglobin content of the supernatant fluid. There were no detectable amounts of haemoglobin in the various samples and it may be presumed therefore that the amount of residual blood in the organs was negligible.

RESULTS.

Our results confirm the earlier observations of Rajam *et al.* (1958) that ascites tumour cells can be labelled with radioactive chromium. According to Rajam, the excess radiochromate in the incubated cell suspension is washed out after three consecutive washings, and the uptake of radioactivity when 10 μc of $Na_2Cr^{51}O_4$ is added to 10^7 cells is 47·5 p.c.

In the present experiments, however, it was necessary to wash the incubated cell suspensions at least five times before "plateau" values were reached in the supernatant fluid (Fig. 1). The uptake of radiochromate did not then exceed 33·3 p.c. (Table 1). By adding lesser amounts of radioactivity (5 μc to 10^7 cells) the uptake was increased by 5 p.c. (Table 1).

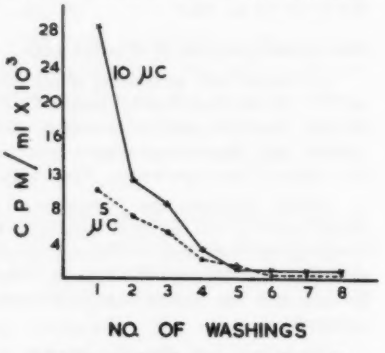
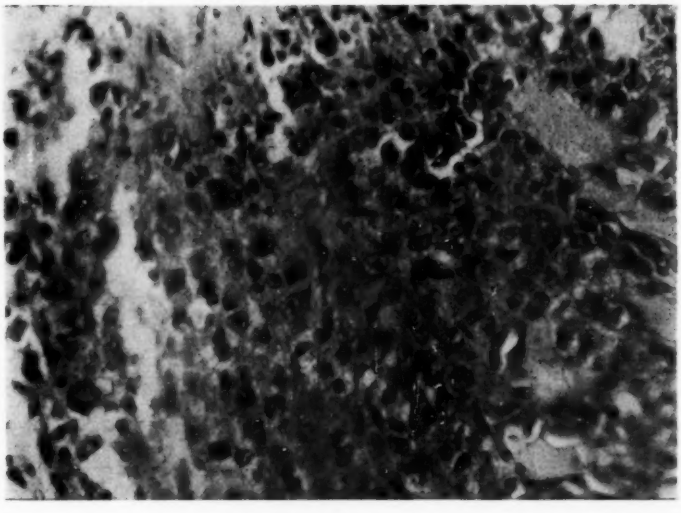


Fig. 1. The decrease in the radioactivity of the supernatant fluid of the labelled ascites tumour cell suspension following cell washing with physiological saline.

Viability of cells.

The viability of the cells labelled in the above described quantities and concentrations was shown by their capacity to produce solid subcutaneous growths in a period of 10 to 12 days after inoculation (Figs. 2a and 2b). However, the ability of these cells to form blood-borne metastases as compared with unlabelled cells (Warren *et al.*, 1936) needs further investigation.

a.



b.

Fig. 2. *a.* A subcutaneous tumour growth obtained ten days after inoculation of $1{\cdot}5 \times 10^6$ labelled cells.

b. Histological section of the subcutaneous growth showing numerous mitotic figures and invasion of the underlying muscle strips.

TABLE 1.

Removal of excess radiochromate by saline washing of tumour cells.

	Added radiochromate	
	10 μc/10^7 cells	5 μc/10^7 cells
Before washing	91,305 c.p.m./ml.	47,511 c.p.m./ml.
Supernatant:		
1	28,634 c.p.m./ml.	10,077 c.p.m./ml.
2	11,190 c.p.m./ml.	7,043 c.p.m./ml.
3	8,614 c.p.m./ml.	5,535 c.p.m./ml.
4	3,222 c.p.m./ml.	2,304 c.p.m./ml.
5	1,160 c.p.m./ml.	1,608 c.p.m./ml.
6	936 c.p.m./ml.	513 c.p.m./ml.
7	882 c.p.m./ml.	584 c.p.m./ml.
8	908 c.p.m./ml.	566 c.p.m./ml.
Total washed cells	30,435 c.p.m./ml.	17,921 c.p.m./ml.
Percentage uptake radiochromate by cells	33·3	37·7

Distribution and localisation of labelled cells.

The distribution and localisation of labelled cells in different organs at various time intervals is shown in Table 2 and graphically in Fig. 3. At the first reading, five minutes after the injection of $1{\cdot}5 \times 10^6$ labelled cells, about 28·6 p.c. of the cells had lodged in the lungs while 29 p.c. remained in the blood stream. It is interesting to note that at that time about 7·8 p.c. of the cells were already lodged in the liver, kidneys and spleen.

Blood stream: There is a rapid rate of removal of tumour cells from the blood stream. After 15 minutes only about 12·1 p.c. of the injected cells are still present in the blood. A further drop to 4·8 p.c. is seen after 30 minutes and there is no detectable amount of radioactivity in the blood stream after six hours (Fig. 3 and Table 2).

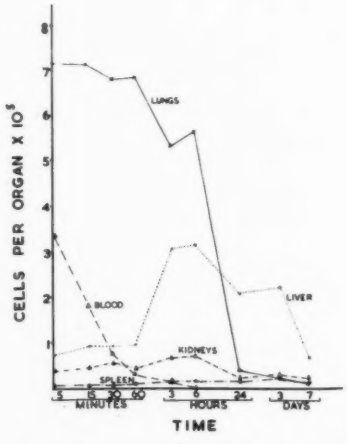


Fig. 3. The distribution of labelled cells in different organs at various time intervals after the intravenous injection of $1{\cdot}5 \times 10^6$ labelled ascites tumour cells. Time is plotted on a logarithmic scale.

Lung: The rapid removal of tumour cells from the blood stream during the first 15 minutes after injection is accompanied by a sharp rise in the number of cells lodged in the lungs. At the end of this time the concentration of cells in the lungs reaches its maximum, the lungs then containing about 47·6 p.c.

of the cells injected (Fig. 3 and Table 2). A decrease from this maximum then occurs, but in the first three hours the decrease is relatively slight. It is apparent from the blood curve that the number of cells in the lungs does not start to fall rapidly until all injected cells have been removed from the circulation (Fig. 3). At the end of 24 hours few cells remain in the lungs (5·4 p.c. of the 15-minute reading).

There is a simultaneous rise in the number of tumour cells in the liver, kidneys and spleen during the first six hours after injection. At the end of this time the concentration of cells in the liver and kidneys reaches its highest level. It is likely that the constant release of cells from the lungs accounts for the rise in the number of cells in these organs.

Liver: Next to the lungs, the liver shows the highest concentration of tumour cells. At the end of six hours about 20 p.c. of the cells injected are in the liver. During the next three days only about 33 p.c. of the accumulated cells disappear from this organ. At the end of one week the total number of cells present in the liver is still the highest of any organ investigated.

Kidneys: The kidneys show marked variation in the rate of accumulation and removal of cells during the first hour. At the end of six hours the greatest number of cells can be detected (about 5 p.c. of the total number injected). The fall noted at 24 hours is more marked than in the liver and about 66 p.c. of the cells accumulated in the first six hours have disappeared. Statistically significant variations in the numbers of cells accumulated and removed from the kidneys were noted, also between 24 hours and one week after injection. The graph thus produced gives an impression of marked fluctuations in the rate of accumulation and removal of cells in the kidney.

Spleen: The spleen shows a constant, steady accumulation of cells during the first three days and about 1·6 p.c. of the injected cells can be detected at this time in this organ. At one week the decline is a moderate one and only 23·5 p.c. of the accumulated cells have been lost. It should be noted that the concentration of cells per gram of splenic tissue at 24 hours is higher than that of the kidneys or liver, and remains so up to the end of the experiment.

DISCUSSION.

The nature of the chromium labelling of tumour cells is not clear, but apparently the chromium unites firmly with some intracellular constituent. Only a small percentage of the chromium is eluted each day and there is apparently no substantial damage to the cell or impairment of its viability. The technique offers a new approach to the problem of the transport and distribution of tumour cell emboli in the blood stream.

Some points of general interest from the present work are as follows:—

(1) The pulmonary-vascular system exerts a significant obstructive force on the circulating cells, so that about half the cells injected are arrested in the lungs during the first 15 minutes. However, immediate transpulmonary passage of tumour cells is evident from the appearance of detectable amounts of radio-

TABLE 2.

Distribution of labelled ascites tumour cells following intravenous injection of $1 \cdot 5 \times 10^6$ cells.
(Mean of 3, 4 or 5 animals ± standard deviation.)

PART I.

Organ	Time interval after injection							
	15 minutes	30 minutes	1 hour	3 hours	6 hours	24 hours	3 days	1 week
(a) CELLS PER WHOLE ORGAN								
Lungs	$7 \cdot 1 \times 10^5$ $\pm 1 \cdot 4 \times 10^5$	$6 \cdot 8 \times 10^5$ $\pm 0 \cdot 5 \times 10^5$	$6 \cdot 9 \times 10^5$ $\pm 0 \cdot 5 \times 10^5$	$5 \cdot 3 \times 10^5$ $\pm 0 \cdot 4 \times 10^5$	$5 \cdot 7 \times 10^5$ $\pm 0 \cdot 97 \times 10^5$	$4 \cdot 0 \times 10^4$ $\pm 1 \cdot 1 \times 10^4$	$2 \cdot 2 \times 10^4$ $\pm 0 \cdot 54 \times 10^4$	$6 \cdot 0 \times 10^3$ $\pm 2 \cdot 9 \times 10^3$
	$\cdot 6 > P > \cdot 5$	$\cdot 9 > P > \cdot 8$	$\cdot 02 > P > \cdot 01$	$\cdot 7 > P > \cdot 6$	$P < \cdot 001$	$\cdot 3 > P > 0 \cdot 2$	$\cdot 2 > P > \cdot 1$	
Livers	$9 \cdot 4 \times 10^4$ $\pm 2 \cdot 5 \times 10^4$	$9 \cdot 3 \times 10^4$ $\pm 2 \cdot 1 \times 10^4$	$9 \cdot 6 \times 10^4$ $\pm 3 \cdot 7 \times 10^4$	$3 \cdot 0 \times 10^5$ $\pm 0 \cdot 34 \times 10^5$	$3 \cdot 2 \times 10^5$ $\pm 0 \cdot 38 \times 10^5$	$2 \cdot 0 \times 10^5$ $\pm 0 \cdot 23 \times 10^5$	$2 \cdot 3 \times 10^5$ $\pm 0 \cdot 71 \times 10^5$	$6 \cdot 7 \times 10^4$ $\pm 1 \cdot 7 \times 10^4$
	$P > \cdot 9$	$P > \cdot 9$	$P < \cdot 001$	$\cdot 8 > P > \cdot 7$	$\cdot 02 > P > \cdot 01$	$\cdot 7 > P > \cdot 6$	$\cdot 02 > P > \cdot 01$	
Kidneys	$4 \cdot 7 \times 10^4$ $\pm \cdot 99 \times 10^4$	$5 \cdot 6 \times 10^4$ $\pm \cdot 64 \times 10^4$	$4 \cdot 5 \times 10^4$ $\pm 1 \cdot 4 \times 10^4$	$6 \cdot 9 \times 10^4$ $\pm 1 \cdot 5 \times 10^4$	$7 \cdot 2 \times 10^4$ $\pm 1 \cdot 7 \times 10^4$	$2 \cdot 6 \times 10^4$ $\pm 0 \cdot 2 \times 10^4$	$3 \cdot 5 \times 10^4$ $\pm 0 \cdot 5 \times 10^4$	$2 \cdot 6 \times 10^4$ $\pm 0 \cdot 4 \times 10^4$
	$\cdot 2 > P > \cdot 1$	$\cdot 3 > P > \cdot 2$	$\cdot 2 > P > \cdot 1$	$\cdot 8 > P > \cdot 7$	$\cdot 01 > P > \cdot 001$	$\cdot 1 > P > \cdot 05$	$\cdot 4 > P > \cdot 3$	
Spleens	$5 \cdot 6 \times 10^3$ $\pm 1 \cdot 6 \times 10^3$	$8 \cdot 9 \times 10^3$ $\pm 3 \cdot 0 \times 10^3$	$1 \cdot 0 \times 10^4$ $\pm \cdot 29 \times 10^4$	$1 \cdot 7 \times 10^4$ $\pm \cdot 54 \times 10^4$	$1 \cdot 7 \times 10^4$ $\pm \cdot 11 \times 10^4$	$1 \cdot 6 \times 10^4$ $\pm \cdot 45 \times 10^4$	$2 \cdot 6 \times 10^4$ $\pm \cdot 85 \times 10^4$	$1 \cdot 7 \times 10^4$ $\pm \cdot 46 \times 10^4$
	$\cdot 2 > P > \cdot 1$	$\cdot 6 > P > \cdot 5$	$\cdot 3 > P > \cdot 2$	$P > \cdot 9$	$\cdot 9 > P > \cdot 8$	$\cdot 2 > P > \cdot 1$	$\cdot 2 > P > \cdot 1$	
Blood	$1 \cdot 8 \times 10^5$ $\pm \cdot 22 \times 10^5$	$7 \cdot 3 \times 10^4$ $\pm 1 \cdot 68 \times 10^4$	$3 \cdot 2 \times 10^4$ $\pm \cdot 34 \times 10^4$	$1 \cdot 5 \times 10^4$ $\pm \cdot 34 \times 10^4$				
	$\cdot 01 > P > \cdot 001$	$\cdot 02 > P > \cdot 01$	$\cdot 01 > P > \cdot 001$					

TABLE 2—*continued.*

PART II.

(b) CELLS PER GRAM ORGAN								
Lungs	$7{\cdot}0\times10^{6}$ $\pm{\cdot}57\times10^{6}$	$7{\cdot}4\times10^{6}$ $\pm{\cdot}44\times10^{6}$	$6{\cdot}7\times10^{6}$ $\pm{\cdot}27\times10^{6}$	$4{\cdot}6\times10^{6}$ $\pm{\cdot}33\times10^{6}$	$3{\cdot}6\times10^{6}$ $\pm{\cdot}11\times10^{6}$	$4{\cdot}7\times10^{5}$ $\pm{\cdot}62\times10^{5}$	$1{\cdot}9\times10^{5}$ $\pm{\cdot}38\times10^{5}$	$3{\cdot}8\times10^{4}$ $\pm{\cdot}4\times10^{4}$
	$\cdot2>P>\cdot1$	$\cdot05>P>\cdot02$	$\cdot01>P>\cdot001$	$\cdot01>P>\cdot001$	$\cdot02>P>\cdot01$	$\cdot01>P>\cdot001$	$\cdot01>P>\cdot001$	
Livers	$1{\cdot}0\times10^{5}$ $\pm{\cdot}17\times10^{5}$	$1{\cdot}2\times10^{5}$ $\pm{\cdot}13\times10^{5}$	$1{\cdot}5\times10^{5}$ $\pm{\cdot}031\times10^{5}$	$3{\cdot}3\times10^{5}$ $\pm{\cdot}42\times10^{5}$	$4{\cdot}1\times10^{5}$ $\pm{\cdot}4\times10^{5}$	$2{\cdot}8\times10^{5}$ $\pm{\cdot}51\times10^{5}$	$2{\cdot}8\times10^{5}$ $\pm{\cdot}28\times10^{5}$	$8{\cdot}1\times10^{4}$ $\pm1{\cdot}0\times10^{4}$
	$\cdot8>P>\cdot7$	$\cdot01>P>\cdot001$	$\cdot01>P>\cdot001$	$\cdot1>P>\cdot05$	$\cdot05>P>\cdot02$	$\cdot5>P>\cdot4$	$P<\cdot001$	
Kidneys	$1{\cdot}9\times10^{5}$ $\pm{\cdot}29\times10^{5}$	$3{\cdot}2\times10^{5}$ $\pm{\cdot}37\times10^{5}$	$3{\cdot}2\times10^{5}$ $\pm{\cdot}06\times10^{5}$	$3{\cdot}3\times10^{5}$ $\pm{\cdot}62\times10^{5}$	$3{\cdot}6\times10^{5}$ $\pm{\cdot}52\times10^{5}$	$1{\cdot}7\times10^{5}$ $\pm{\cdot}08\times10^{5}$	$1{\cdot}8\times10^{5}$ $\pm{\cdot}15\times10^{5}$	$1{\cdot}4\times10^{5}$ $\pm{\cdot}2\times10^{5}$
	$P<\cdot001$	$P>\cdot9$	$\cdot3>P>\cdot2$	$\cdot6>P>\cdot5$	$\cdot01>P>\cdot001$	$\cdot8>P>\cdot7$	$\cdot05>P>\cdot02$	
Spleens	$9{\cdot}9\times10^{4}$ $\pm2{\cdot}0\times10^{4}$	$1{\cdot}4\times10^{5}$ $\pm{\cdot}18\times10^{5}$	$1{\cdot}4\times10^{5}$ $\pm{\cdot}2\times10^{5}$	$2{\cdot}0\times10^{5}$ $\pm{\cdot}5\times10^{5}$	$1{\cdot}7\times10^{5}$ $\pm{\cdot}08\times10^{5}$	$4{\cdot}0\times10^{5}$ $\pm{\cdot}8\times10^{5}$	$4{\cdot}3\times10^{5}$ $\pm{\cdot}22\times10^{5}$	$2{\cdot}1\times10^{5}$ $\pm{\cdot}62\times10^{5}$
	$\cdot01>P>\cdot001$	$\cdot9>P>\cdot8$	$\cdot2>P>\cdot1$	$\cdot6>P>\cdot5$	$\cdot01>P>\cdot001$	$\cdot7>P>\cdot6$	$\cdot01>P>\cdot001$	
Blood	$4{\cdot}4\times10^{5}$ $\pm{\cdot}23\times10^{5}$	$1{\cdot}5\times10^{5}$ $\pm{\cdot}18\times10^{5}$	$9{\cdot}6\times10^{4}$ $\pm{\cdot}5\times10^{4}$	$3{\cdot}1\times10^{4}$ $\pm{\cdot}48\times10^{4}$				
	$P<\cdot001$	$\cdot01>P>\cdot001$	$P<\cdot001$					

activity in the organs supplied by the systemic circulation as early as five minutes after the injection. This observation favours the existence of large arterial-venous shunts in the pulmonary-vascular bed (Prinzmetal, Ornitz, Simkin and Bergman, 1948).

(2) Only 5 p.c. of the number of cells found in the lungs 15 minutes after injection were still found in these organs at the end of 24 hours. It follows that only a few of the cells reaching the lungs from a tumour can produce metastatic growths in the lungs.

(3) About 20 p.c. of the injected cells were found in the liver after six hours and the number remained high for some days. It is therefore not surprising that metastases are so commonly found in the liver. The present findings also suggest that at least some liver metastases may be the result of transpulmonary passage of embolic cells entering the systemic circulation, although the majority probably do occur through the portal venous system or by lymphatic spread.

(4) It is interesting to note, in view of the rarity of metastases in the spleen in human neoplasms, that the total number of cells reaching this organ is small. However, the concentration of cells per gram of tissue is high and at the end of 24 hours exceeds the corresponding concentration in the liver. The rate of removal or disintegration of cells is slow. These observations suggest that a distributional factor is unlikely to be responsible for the rarity of metastasis in the spleen.

These studies indeed suggest that in the case of ascites tumour cells in the mouse at least three factors are involved in the growth of metastases: (1) the distribution of tumour cells to the various organs, (2) the ability of the cells to implant themselves in the organ, and (3) their ability to grow after implantation. While the transport factor is obviously of great importance, the local environment must play a rôle if and when the cell is trapped in any situation in the tissues.

Acknowledgments. The author wishes to acknowledge the assistance given to him by Dr. R. J. Walsh, Director of the New South Wales Red Cross Blood Transfusion Service, who supervised the work; and by Dr. F. F. Rundle, Head of the Unit of Clinical Investigation, Royal North Shore Hospital.

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PLASMA CELL PROLIFERATION FOLLOWING WHOLE BODY IRRADIATION

by G. J. V. NOSSAL[1, 2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

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SUMMARY.

The effects of large doses of whole body irradiation on lymphoid tissues have been studied, with special reference to the normal plasma cell populations of the tissues. Plasma cells were found to be more radioresistant than lymphocytes.

After a sublethal dose of 500 r of whole body X-rays, considerable plasma cell proliferation followed. This plasma cell proliferation occurred earlier and was relatively more extensive than lymphocyte regeneration.

The view is put forward that the plasma cell proliferation might represent an antibody response to invading micro-organisms entering the irradiated animal *via* the damaged gastrointestinal tract.

INTRODUCTION.

Since Heineke (1905) first described the great radiosensitivity of lymphoid tissues, much work has been done on this problem. The chief findings have been reviewed by Bloom (1948) and more recently by Harris (1958). In most of this work, changes in the lymphocyte population of lymph nodes have been studied in great detail, but plasma cell changes have not been recorded so exactly. As the primary immune response is suppressed by large doses of X-rays one might expect that plasma cell development after irradiation would be absent or limited. Findings consistent with this view were obtained by Fitch, Wissler, La Via and Barker (1956), who noted that the number of pyroninophilic cells developing in the red pulp of the spleen following antigenic stimulation was diminished after whole body irradiation.

In the present study, changes in the plasma cell population of mesenteric and popliteal lymph nodes and thymus in rats after 500-1000 r of whole body X-rays are reported. An extraordinary degree of plasma cell proliferation following 500 r, and prolonged survival of some plasma cells following 1000 r have been found.

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[1] Present address: Department of Genetics, Stanford University, Stanford, California.

[2] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

Materials and Methods.

Animals.

Adult male Wistar albino rats bred by random mating and aged 4-6 months were used. They were fed on a diet of mouse pellets and tap water *ad libitum*.

Irradiation.

Whole body X-irradiation was given in doses of 500 or 1000 r under the following conditions: 250 kv., 15 m.a., 35 cm. distance to the container, yielding 111 r per minute in air. Filtration was by 1 mm. of aluminium and 0·5 mm. of copper, giving a half-value layer of 1·0 mm. of copper.

Bone marrow injections.

In some experiments, rats received injections of normal homologous bone marrow. Adult donor rats were killed and their femurs and tibias removed. Bone marrow was obtained by cutting off the ends of the bones and syringing the shafts through with Earle's saline. Cell clumps were broken up by repeated gentle pipetting, and the bone marrow from one donor was injected intravenously into 1 recipient rat about 2 hr. after irradiation.

Histological techniques.

Rats were killed by exsanguination under chloroform anaesthesia and the mesenteric lymph nodes, both popliteal lymph nodes and thymus were removed. The tissues were placed in Helly's fixative at room temperature for 1 hr. and at 4° C. for 15-20 hr. They were then embedded in paraffin and sections 5 μ thick were prepared by standard histological techniques. They were stained with methyl green-pyronin and mounted in DPX.

In some experiments, single cell suspensions were prepared from mesenteric lymph nodes and air-dried cell smears were stained by Leishman's stain as previously described (Nossal, 1959).

RESULTS.

Definition of a plasma cell.

While there is little doubt about the histological features of a mature plasma cell, there is considerable confusion in the literature regarding the nomenclature of more primitive types of plasma cells. We regard cells of the plasma cell series as being characterized by an eccentric nucleus, strongly basophilic or pyroninophilic cytoplasm and often, though not always, a perinuclear clear zone. We further arbitrarily subdivide the cells into three classes: (a) *plasmablasts*, which are the same size as the normal blast cells or large lymphocytes occurring in the germinal centres of lymph nodes, but differ from them by having more abundant and basophilic (or pyroninophilic) cytoplasm, a slightly denser nuclear chromatin pattern, and a nucleus which is generally placed to one side of the cell and is slightly ovoid or elongated; (b) *immature plasma cells*, which have very abundant cytoplasm which may be foamy in smears, and a smaller, denser, distinctly eccentric nucleus; (c) *mature plasma cells*, which are smaller, whose cytoplasm is less abundant, and whose nucleus is smaller and denser. In a good histological preparation, cells of the plasma cell series are distinguished with ease from primitive lymphoid forms.

Plasma cell development in normal tissues.

Sections and smears of cells from many normal adult rats were examined. In the mesenteric lymph nodes, there were always many plasma cells. These occurred in the medulla in groups and cords of some dozens of cells. Sometimes whole sheets of plasma cells comprising hundreds of cells were seen deep in the node. In smears, the number of plasma cells was about 2 p.c. of the total cell content of the node.

In normal popliteal lymph nodes, plasma cells were much less frequent. They occurred singly or in small groups in the medullary cords. In smears, they generally comprised 0·2 to 0·5 p.c. of the total cell population, and were somewhat less frequent than large lymphocytes (blasts).

In normal thymuses, plasma cells were very infrequent. Occasionally one could be seen right at the periphery of the cortex immediately under the capsule.

Effects of 500 r whole body irradiation.

After 500 r, plasma cells were more radioresistant than lymphocytes. In the mesenteric nodes 1 day after irradiation, there were virtually no lympho-

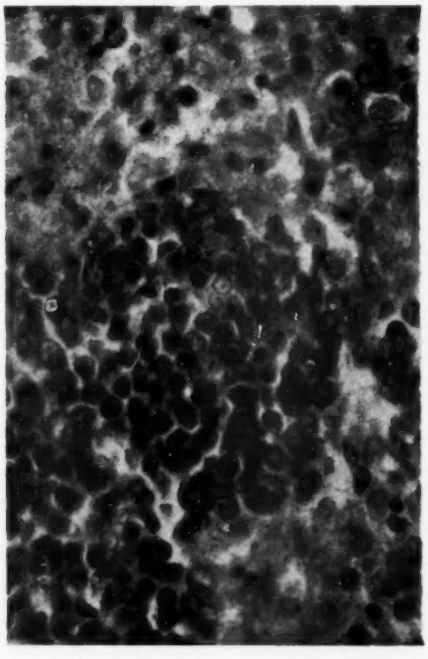

Fig. 1. Section of mesenteric node 5 days after 500 r. Note the mitotic figures amongst plasmablasts and immature plasma cells. x 768.

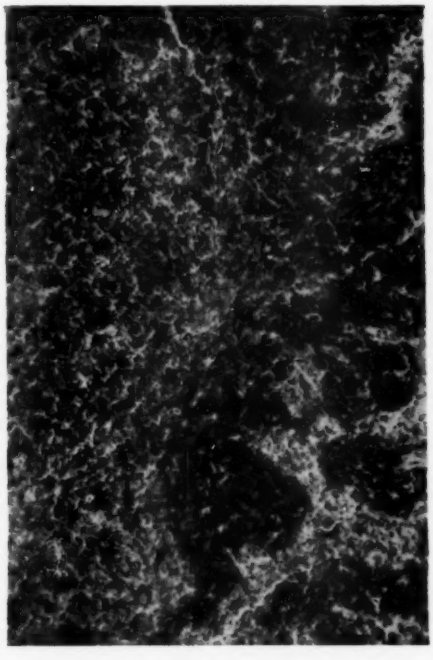

Fig. 2. Section of mesenteric node 10 days after 500 r. Note plasma cell proliferation (right) and diffuse lymphoid proliferation (left). x 144.

cytes left, leaving the cortex a depleted connective tissue reticulum occupied only by macrophages. At this stage, the number of plasma cells appeared to be within normal limits. By 3 days, the number of plasma cells was slightly but definitely reduced, although a few mitotic figures were apparent in the remaining cells even at this stage. No lymphocyte regeneration was evident in the cortex. By 4 and 5 days (Fig. 1) mitosis was proceeding rapidly amongst plasmablasts and immature plasma cells. By 5 days, some diffuse cortical regeneration of large and medium lymphocytes was also apparent. By 7 days, marked plasma cell proliferation in the medulla was accompanied by marked diffuse lymphocyte regeneration in the cortex. By 10 days (Fig. 2), medullary plasma cell proliferation was at its height, and very large areas of the nodes were occupied by plasma cells. Nodule formation was commencing in the cortex, and in smears the large numbers of plasma cells and immature lymphoid forms was in marked contrast to the paucity of small lymphocytes. Some diffuse haemorrhage was also apparent at this stage. By 14 days, haemorrhage was slightly more marked and follicular regeneration of lymphocytes was at its height. There were still no small lymphocytes cuffing the germinal centres. In the medulla, most of the cells seen were mature plasma cells. By 19 days, the relative area occupied by

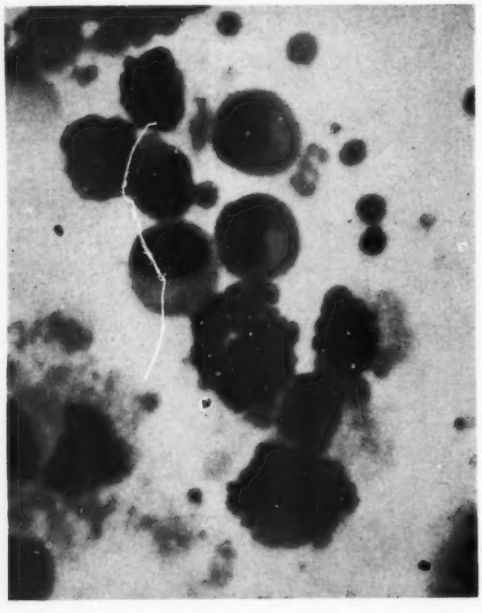

Fig. 3. Smear of mesenteric node 7 days after 1000 r. Note the plasma cells of normal appearance and numerous smeared cells. x 2000.

plasma cells was much less, and the germinal centres, while still very active, now had narrow cuffs of small lymphocytes around them. By 24 days, while the number of small lymphocytes and the total thickness of the cortex were still reduced the general architecture was approaching normal. By 37 and 45 days, apart from some thinning of the cortex, the nodes appeared normal.

Effects of 1000 *r whole body irradiation on plasma cells.*

After 1000 r, the effects of irradiation on both lymphocytes and plasma cells were more profound than after 500 r. All the rats X-rayed but not injected with bone marrow died within the first 7 days and over this period there were no differences between sections of their organs and sections from bone marrow-injected rats. The most striking feature of the sections after 1000 r was the great difference in radiosensitivity of lymphocytes and plasma cells. Sections taken between 1 day and 9 days after irradiation all showed persistence of plasma cells but complete absence of lymphocytes. However, a gradual diminution of the number of plasma cells over this period was apparent. One day after irradiation, plasma cells still occupied considerable areas of mesenteric node sections. By 2 days, macrophages were seen scattered amongst the plasma cells and some plasma cells exhibited pyknotic nuclei and fragmented cytoplasm. This degeneration of plasma cells continued progressively until 9 days. At this stage, plasma cells were seen in small collections of up to 20 cells only, and many of them were necrotic. From then on, there was a steady but very slow increase in the number of plasma cells. From the 5th to the 9th day, further interesting features were considerable haemorrhage into the nodes, more marked in mesenteric than popliteal, disappearance of macrophages from the cortex and marked destruction of the connective tissue reticulum. Over this period, the persisting plasma cell areas stood out prominently as the only portions of the nodes with some intact cells (Fig. 3). After 12 days, progressive diffuse regeneration was seen, but even after 30 days, the thickness of the cortex and the number of plasma cells in the medulla were somewhat reduced.

The effects on the thymus with 1000 r were even more complete than on the lymph nodes. In the first 12 days, there was no regeneration of lymphocytes. However, a few plasma cells were seen in all thymuses both immediately under the capsule and deeper in the cortex. It is possible that there were a few more plasma cells present than in normal thymuses, but this is difficult to assess as plasma cells present in normal thymuses may have been obscured by the large number of lymphocytes densely packing the cortex. After 12 days, rapid repopulation of the thymus occurred so that by 30 days, the thymus was virtually normal.

DISCUSSION.

Though a voluminous literature exists on the histological effects of irradiation, the detailed effects of irradiation on normal plasma cell populations have apparently not been fully recorded. In a short communication, Hill (1959)

has stated that plasma cells occupied large areas of lymph nodes following irradiation. We have independently observed an extraordinary degree of plasma cell proliferation after sublethal doses of whole body irradiation. After 500 r, there was a slight diminution in plasma cell numbers over the first three days, followed by extensive proliferation for at least 2 weeks. After 100 r, though there was definite gradual destruction of plasma cells, this proceeded much more slowly than lymphocyte destruction, and considerable numbers of intact plasma cells were seen at times when there were no lymphocytes whatever present in the nodes. However, after 1000 r, lymphocyte regeneration in the early recovery phase began earlier and was more rapid than plasma cell regeneration.

Plasma cells are generally considered to be the producers of circulating antibody (Leduc, Coons and Connolly, 1955; Nossal, 1959) and the relative radioresistance of plasma cells is consistent with the observation that established antibody production is not greatly affected by irradiation (Taliaferro and Taliaferro, 1951). The most probable explanation for the plasma cell proliferation following 500 r is that the cells may have been forming antibody to the large variety of micro-organismal antigens entering the body from the damaged gastrointestinal tract. This seems a particularly satisfactory explanation of the proliferation in mesenteric nodes. As some antigenic material probably entered from the intestine in normal animals, cells in the mesenteric nodes, and possibly some cells in other regions might have had experience of many of the antigens before irradiation. The post-irradiation plasma cell proliferation would then have represented a specific anamnestic response, which is known to be more radioresistant than a primary response (Dixon, Talmage and Maurer, 1952; Taliaferro, 1957). Among other possibilities this may be a non-specific proliferative process bearing no relation to antigenic stimulation and producing either a wide range of antibody of types dependent on the previous experience of the animal, or conceivably protein without immunological specificity.

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THE ESTABLISHMENT OF A STRAIN OF HUMAN CELLS IN TISSUE CULTURE

by MAUREEN F. LARKIN

(From the New South Wales State Cancer Council, Special Unit, Prince of Wales Hospital, Randwick, N.S.W.).

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SUMMARY.

A strain of human cells—B-184—has been grown continuously in tissue culture for over 12 months. The cells are maintained easily in simple fluid medium on glass. They can be stored successfully at — 50° C. for at least four months. Poliovirus multiplied within these cells and produced cytopathogenic effects.

INTRODUCTION.

In the tissue culture laboratory of the Special Unit, it has been the practice to culture all suitable specimens removed at operation from patients with cancer. The purpose of this work was to try to establish different strains of cells for comparative and chemical studies. The technique used has varied. Sometimes fragments of the tissue have been cultured alone and in other cases, cell suspensions of the tumour have been used in addition. Sometimes a solid substrate was provided in the form of a plasma clot (Robbins, Weller and Enders, 1952a) in which the cells or fragments were embedded to see if this would give more satisfactory results. Various types of media, both biological and synthetic, were tried.

Various investigators have reported the development of strains of human cells, but in many instances these strains could not be kept continuously in culture. Thus, relatively few lines of human cells have been established (Chang, 1954; Leighton, 1954; Orr and McSwain, 1955; Berman and Stulberg, 1956; Gey, Coffman and Kubicek, 1952). Our results show that of 190 specimens received it was possible with approximately 50 p.c. to obtain satisfactory growth and survival after one or two passages, but in all cases except the one which will now be described, the cells were eventually lost when transplantation was attempted using either mechanical procedures or Bacto-Trypsin 1:250 (Difco) in concentrations from 0·01 to 0·5 p.c.

The present report is concerned with a history of the cell strain, procedures used for its isolation and maintenance, and observations on its morphology and growth behaviour.

Materials and Methods.

B-184 cells were derived from a fifteen-year-old female patient with a giant cell tumour of the lateral condyle of the right femur. Histological sections (Figs. 1 and 2) showed a typical osteoclastoma with giant cells interspersed among sheets of small plump spindle-shaped cells. There were a number of mitotic figures present in the smaller cells. The histological picture was that of the more common type of giant cell tumour. It was considered that this tumour may recur locally, but that distant metastasis was unlikely.

Initial cultures (28th June, 1958) were prepared within 30 minutes of the tumour being removed from the body. The fragments, approximately 1 $mm.^3$, were grown directly on the glass surface of 4-oz. medical flats, using Hank's Salt Solution (H.S.S.) as the dispersing fluid. After the fragments had adhered to the glass, 6 ml. of medium were added and the bottles incubated at 37° C. in a horizontal position. The medium consisted of H.S.S. 55 p.c., horse serum 40 p.c. (Robbins, Weller and Enders, 1952b), chick embryo extract 5 p.c. (Younger, Ward and Salk, 1952) with 100 units of penicillin and 0·1 mg. of streptomycin per ml. The medium was changed twice weekly: up to 50 p.c. of the old medium being retained at each change.

After approximately 130 days in culture at which time the cells were in their 5th passage, a deterioration in their nutritional state was evident (Figs. 3 and 4). The cultures became more granular and fewer cells survived trypsinization. At this stage the medium was changed to H.S.S. 66 p.c., pooled human serum 30 p.c., 5 p.c. lactalbumin hydrolysate 4 p.c., and within a week the cells had resumed their normal, healthy appearance (Figs. 5 and 6). In addition, the proliferation rate had increased necessitating transfers at 6-8-day intervals as against the previous interval of 20-30 days. In this time a fivefold increase in the number of cells is usually obtained. This medium is now used routinely for this strain.

Transfers.

Transfers can be made using a 0·25 p.c. solution of Trypsin (Dulbecco and Vogt, 1954). The old medium is removed, centrifuged at 2000 r.p.m. for five minutes and mixed with an equal part of fresh medium to form the nutritive solution for each transfer generation. The bottles, containing 4 ml. of trypsin, are incubated at 37° C. for 10-15 minutes to detach the cells from the glass surface. This cell suspension is then centrifuged at 1000 r.p.m. for five minutes, the supernatant discarded and the cells re-suspended in 5 ml. of the nutritive solution. Cell counts are made using an haematocytometer and the suspension diluted so that each ml. contains 1×10^5 cells. Fresh bottles are then inoculated with this suspension and incubated at 37° C.

Preservation of cells in frozen state.

In view of the fact that strain HeLa and Earle's strain L-cells will withstand freezing at sub-zero temperatures (Scherer and Hoogasian, 1954) an attempt was made to store B-184 cells in the frozen state. Solutions of glycerol (5 p.c.-30 p.c.) in nutritive solution, in Hank's salt solution and in pooled human serum were tried, but the only combination giving good preservation of the cells was 5 p.c. glycerol in pooled human serum. The cells were sealed in 5 ml. ampoules and frozen within 45 minutes at — 50° C.

When re-culturing, the ampoule is placed in a 37° C. water bath and the cells thawed within three minutes. The cell suspension is removed to a sterile centrifuge tube, the ampoule rinsed once with H.S.S., added to the cell suspension and centrifuged at 1000 r.p.m. for five minutes. Cell counts are made in the same manner as before, but the size of the inoculum is increased to 3×10^5 cells per ml. This is to allow for those cells which have not survived freezing and thawing and those which were dead before freezing. The nutritive medium contains 50 p.c. old medium. By this method B-184 cells have been successfully re-cultured after a period of four months.

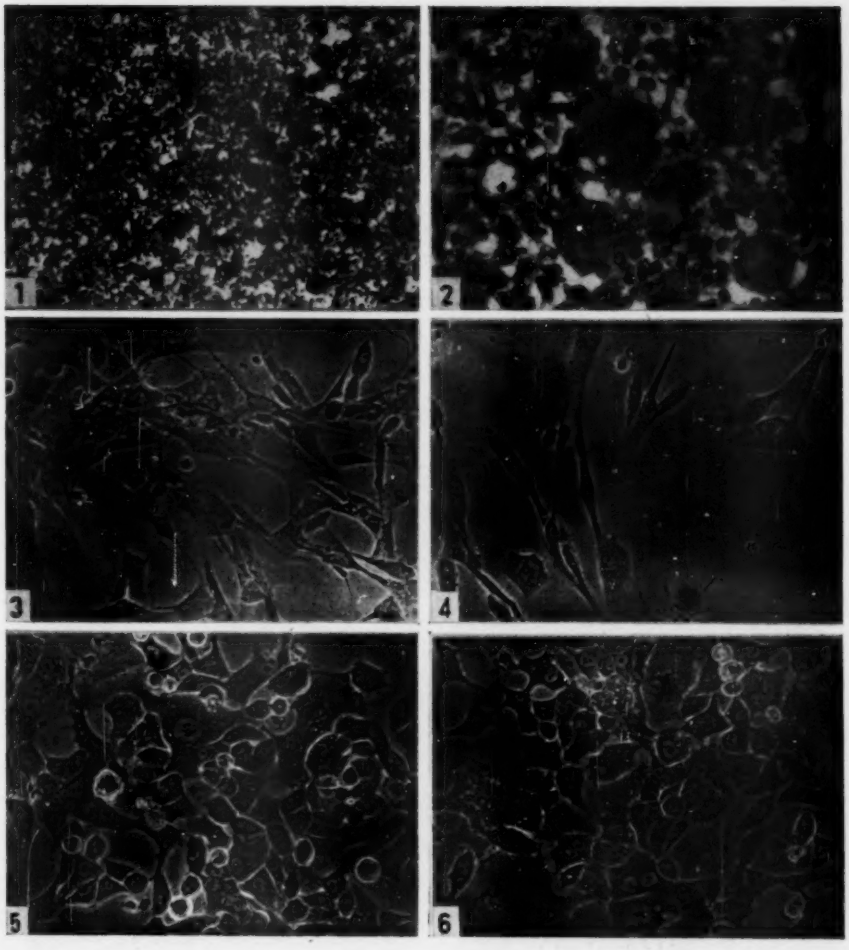

Fig. 1. Section of giant cell tumour. Haematoxylin and Eosin. Phase contrast x 133.

Fig. 2. Same section. Higher magnification x 400.

Fig. 3. 17-day-old culture of B-184 cells. Phase contrast x 133.

Fig. 4. B-184 cells after 120 days in culture (P.4). The cells in Figs. 3 and 4 were cultured in H.S.S., H.S., and C.E.E. Phase contrast x 133.

Fig. 5. Same cells after 160 days (P.8) in culture. Phase contrast x 133.

Fig. 6. Same cells after 250 days (P.20) in culture. Cells in Figs. 5 and 6 were cultured in H.S.S., PHuS, and L.A.H. Phase contrast x 133.

RESULTS.

After distribution of the suspension in the culture bottles, the cells settle on the glass surface within a few hours. Some of the cells do not attach themselves to the glass. These are probably the ones that die during the process of transfer. These, together with the resulting debris, are gradually eliminated when the medium is changed.

Within 24 hours the cells become flattened and by 6-8 days have multiplied to form an epithelial sheet. These sheets are composed of a single layer of cells of various sizes and shapes. Many of the cells are multinucleated and many of the nuclei have more than one nucleolus. The cytoplasm is often granular.

In the early stages, the cultures were composed of giant cells and smaller spindle-shaped cells; but after several transfers the giant cells disappeared, either because the medium was not suitable for them or because they were overgrown by the more active smaller cells undergoing continuous proliferation. These smaller cells have now become more polyhedral in shape and progressively more homogeneous in structure. Several time-lapse microcinematographic films have been made and while mitoses were frequent in the smaller cells; no mitotic activity was observed in the giant cells. The average time for mitosis of the smaller cells as calculated from these films was of the order of 80 minutes. The formation of a multinucleated cell by nuclear division without cytoplasmic division of a mononucleated cell was also not observed. Nuclear rotation and cytoplasmic streaming which are seen in films of many cells in culture was also a feature of these cells.

Because of the relative ease with which these cells may be cultured, passaged and preserved it was thought likely that they might respond to poliovirus in a manner similar to other human cell lines. With the co-operation of Mr. D. C. Dorman of the Royal Alexandra Hospital for Children, Camperdown, tests were made using three types of poliovirus—Mahoney (Type 1), MEFI (Type 2), and Saukett (Type 3)—and in each case specific cytopathogenic changes were produced, similar to those seen in HeLa cells.

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